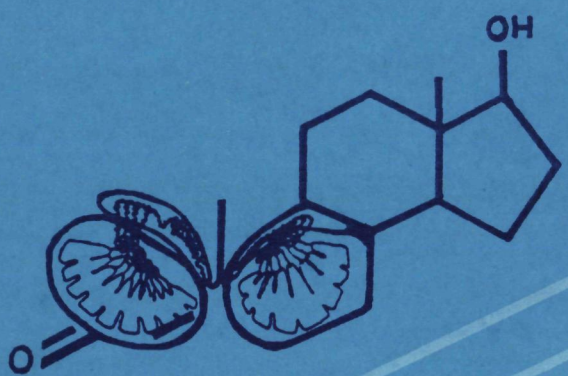
biochemical pathways in human testicular steroidogenesis



jos weusten

BIOCHEMICAL PATHWAYS IN HUMAN TESTICULAR STEROIDOGENESIS

Een wetenschappelijke proeve op het gebied
van de Geneeskunde en Tandheelkunde

Proefschrift

ter verkrijging van de graad van doctor aan de
Katholieke Universiteit te Nijmegen, volgens
besluit van het college van decanen in het
openbaar te verdedigen op vrijdag 10 maart
1989 des namiddags om 1.30 uur precies

door

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Geboren op 1 juli 1962 te Boxtel

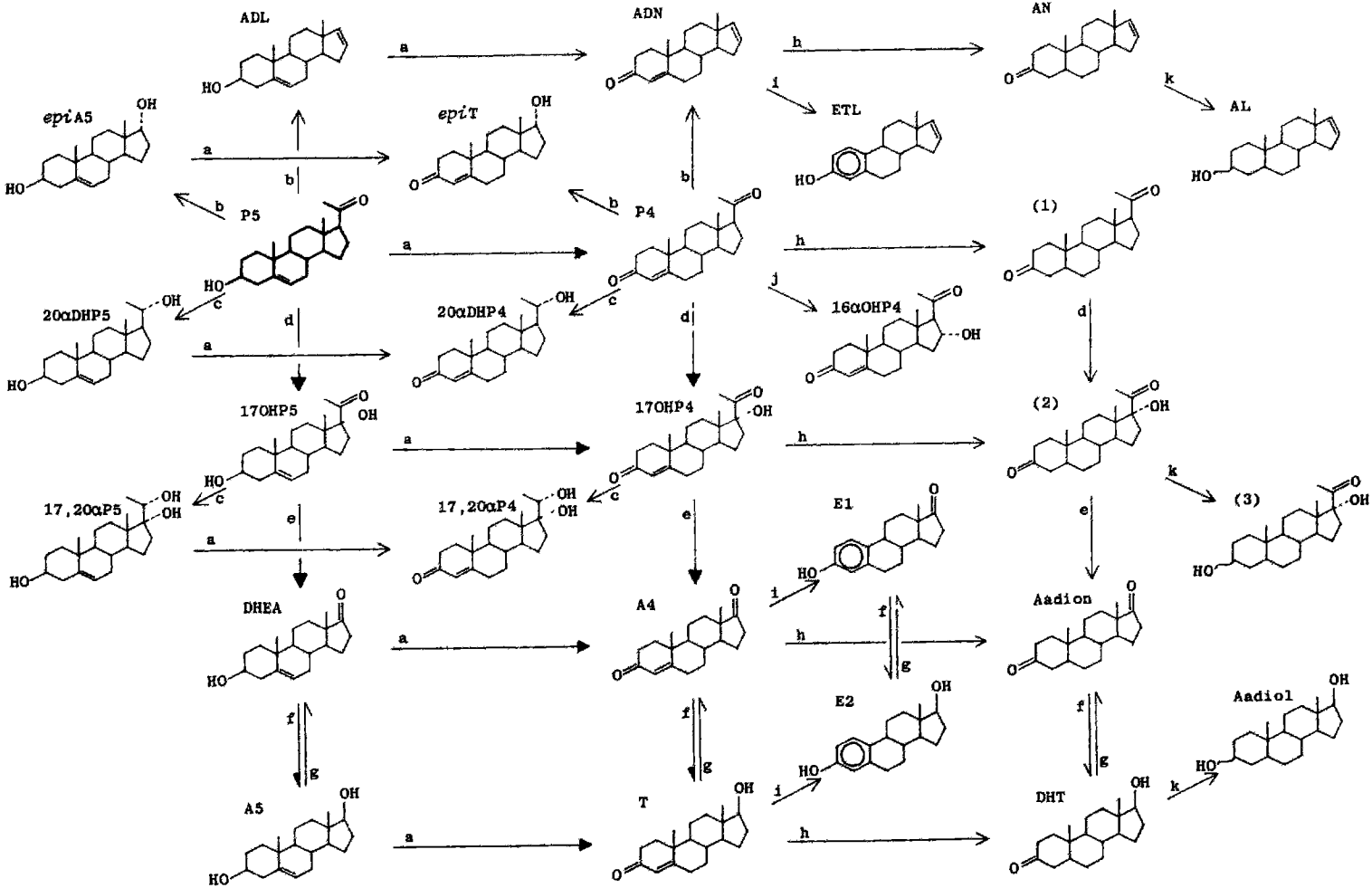
Pressa Trajectina, Utrecht

Promotores: Prof. Dr. A.G.H. Smals
Prof. Dr. Th.J. Benraad

The investigations presented in this thesis were performed in the Division of Endocrinology (Head: Prof. Dr. P.W.C. Kloppenborg), Department of Medicine (Head: Prof. Dr. A. van 't Laar), and the Department of Experimental and Chemical Endocrinology (Head: Prof. Dr. Th.J. Benraad), Sint Radboud Hospital, University of Nijmegen, Nijmegen, The Netherlands.

Aan mijn ouders

BIOCHEMICAL PATHWAYS IN HUMAN TESTICULAR STEROIDOGENESIS


ADL
ADN
AN
epiA5
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P5
P4
(1)
20αDHP5
20αDHP4
16αOHP4
17OHP5
17OHP4
(2)
17,20αP5
17,20αP4
E1
(3)
DHEA
A4
Aadion
E2
Aadiol
A5
T
DHT
a
b
c
d
e
f
g
h
i
j
k

THE MOST IMPORTANT STEROIDS
MENTIONED IN THIS THESIS
AND THEIR BIOSYNTHETICAL
RELATIONS

ENZYMES

a, 3ß-hydroxysteroid dehydrogenase/
Δ5-3-ketosteroid isomerase
b, 16-ene-synthetase
c, 20α-hydroxysteroid dehydrogenase
d, 17α-hydroxylase
e, lyase
f, 17-ketosteroid oxidoreductase
g, 17ß-hydroxysteroid dehydrogenase
h, 5α-reductase
i, aromatase
j, 16α-hydroxylase
k, 3-ketosteroid oxidoreductase

STEROIDS

A4,	androstenedione
A5,	androstenediol
Aadiol,	androstanediols
Aadion,	androstanedione
ADL,	androstadienol
ADN,	androstadienone
AL,	androstenol
AN,	androstenone
DHEA,	dehydroepiandrosterone
20αDHP4,	20α-dihydroprogesterone
20αDHP5,	20α-dihydropregnenolone
DHT,	dihydrotestosterone
E1,	estrone
E2,	estradiol
*epi*A5,	epiandrostenediol
*epi*T,	epitestosterone
ETL,	estratetraenol
16αOHP4,	16α-hydroxyprogesterone
17OHP4,	17-hydroxyprogesterone
17OHP5,	17-hydroxypregnenolone
P4,	progesterone
P5,	pregnenolone
17,20αP4,	17α,20α-dihydroxy-4-pregnen-3-one
17,20αP5,	5-pregnene-3ß,17α,20α-triol
T,	testosterone
(1),	5α-*pregnane*-3,20-dione
(2),	17α-hydroxy-5α-pregnane-3,20-dione
(3),	3ξ,17α-dihydroxy-5α-pregnan-20-one

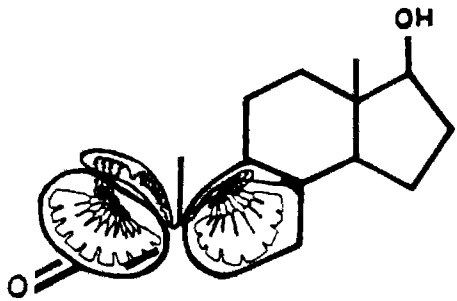
⟶ pathways leading from pregnenolone (P5) to testosterone (T)
⟶ other pathways

LIST OF ABBREVIATIONS AND TRIVIAL NAMES

A4, androstenedione, 4-androstene-3,17-dione
A5, androstenediol, 5-androstene-3ß,17ß-diol
Aadiol, androstanediol, 5α-androstane-3ß,17ß- or -3α,17ß-diol
Aadion, 5α-androstane-3,17-dione
ADL, androstadienol, 5,16-androstadien-3ß-ol
ADN, androstadienone, 4,16-androstadien-3-one
AGI, aminoglutethimide, 3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione; inhibitor of CSCC
AL, androstenol, 5α-androst-16-en-3ß- or -3α-ol
AN, androstenone, 5α-androst-16-en-3-one
androsterone, 3α-hydroxy-5α-androstan-17-one
aromatase; converts A4 to E1 or T to E2
Chol, cholesterol, 5-cholesten-3ß-ol
CK, cyanoketone, win-19578, 2α-cyano-17ß-hydroxy-4,4,17α-trimethyl-5-androsten-3-one; inhibitor of 3ßHSD and 20αHSD
collagenase, clostridiopeptidase A
cpm, counts per minute
CSCC, cholesterol side chain cleavage (complex); converts Chol to P5
cyt.b5, cytochrome b5
cyt.P450, cytochrome P450
16-dehydro-P4, 4,16-pregnadiene-3,20-dione
16-dehydro-P5, 3ß-hydroxy-5,16-pregnadien-20-one
DHEA, dehydroepiandrosterone, 3ß-hydroxy-5-androsten-17-one
20αDHP4, 20α-dihydroprogesterone, 20α(*S*)-hydroxy-4-pregnen-3-one
20αDHP5, 20α-dihydropregnenolone, 5-pregnene-3ß,20α(*S*)-diol
20ßDHP4, 20ß-dihydroprogesterone, 20ß(*R*)-hydroxy-4-pregnen-3-one
20ßDHP5, 20ß-dihydropregnenolone, 5-pregnene-3ß,20ß(*R*)-diol
DHT, dihydrotestosterone, 17ß-hydroxy-5α-androstan-3-one
dpm, desintegrations per minute
E1, estrone, 3-hydroxy-1,3,5(10)-estratrien-17-one
E2, estradiol, 1,3,5(10)-estratriene-3,17ß-diol
16-ene-synthetase; converts 20-keto-C_{21} steroids to 16-androstenes
*epi*A5, 5-androstene-3ß,17α-diol
*epi*T, 17α-hydroxy-4-androsten-3-one
EPOS, epostane, win-32729, 2α-cyano-4α,5α-epoxy-4ß,17α-dimethyl-androstan-17ß-ol-3-one; inhibitor of 3ßHSD and 20αHSD
ETL, estratetraenol, 1,3,5(10),16-estratetraen-3-ol
FCS, fetal calfs serum
GCMS, gass chromatography mass spectrometry
hCG, human chorionic gonadotropin
HPLC, high performance liquid chromatography
hr, hour(s)
3ßHSD, Δ5-3ß-hydroxysteroid dehydrogenase/Δ5-3-ketosteroid isomerase; converts Δ5- to Δ4-steroids
17ßHSD, 17ß-hydroxysteroid dehydrogenase; converts 17ß-hydroxy-C_{19} steroids to 17-keto-C_{19} steroids

20αHSD, 20α-hydroxysteroid dehydrogenase; converts 20α-hydroxy-C_{21} steroids to 20-keto-C_{21} steroids and *vice versa*
3KSOR, 3-ketosteroid oxidoreductase; converts 3-keto-5α steroids to 3α- or 3β-hydroxy-5α-steroids
17KSOR, 17-ketosteroid oxidoreductase; converts 17-keto-C_{19} steroids to 17β-hydroxy-C_{19} steroids
LH, luteinizing hormone
lyase, C17,20-lyase activity of 17αOHase/lyase; converts 17α-hydroxy-20-keto-C_{21} steroids to 17-keto-C_{19} steroids
MEM, minimal essential medium
min, minute(s)
NAD, nicotinamide adenosine dinucleotide
NADH, reduced form of NAD
NADP, nicotinamide adenosine dinucleotide phosphate
NADPH, reduced form of NADP
16αOHase, 16α-hydroxylase; hydroxylates steroids at the 16α-position
17αOHase, 17α-hydroxylase activity of 17αOHase/lyase; hydroxylates 20-keto-C_{21} steroids at the 17α-position
17αOHase/lyase, cyt.P450 with 17αOHase and C17,20-lyase activity
20αOHChol, 20α-hydroxycholesterol, 5-cholestene-3β,20*S*-diol
22*R*OHChol, 22*R*-hydroxycholesterol, 5-cholestene-3β,22*R*-diol
25OHChol, 25-hydroxycholesterol, 5-cholestene-3β,25-diol
16αOHP4, 16α-hydroxyprogesterone, 16α-hydroxy-4-pregnene-3,20-dione
16αOHP5, 16α-hydroxypregnenolone, 3β,16α-dihydroxy-5-pregnen-20-one
17OHP4, 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione
17OHP5, 17α-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one
21OHP4, 21-hydroxyprogesterone, 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione
21OHP5, 21-hydroxypregnenolone, 3β,21-dihydroxy-5-pregnen-20-one
P4, progesterone, 4-pregnene-3,20-dione
P5, pregnenolone, 3β-hydroxy-5-pregnen-20-one
P5i, concentration of P5 as determined via a direct RIA
17,20αP4, 17α,20α(*S*)-dihydroxy-4-pregnen-3-one
17,20αP5, 5-pregnene-3β,17α,20α(*S*)-triol
pregnenediol, 20βDHP5
psi, pounds per square inch
5αRed, 5α-reductase; converts Δ4-3-ketosteroids to 5α-3-ketosteroids
RIA, radioimmunoassay
SD, standard deviation
SU, SU-10603, 7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphtalenone; inhibitor of several cyt.P450 linked enzymes
T, testosterone, 17β-hydroxy-4-androsten-3-one
Ti, concentration of T as determined via a direct RIA
TRIL, trilostane, modrenal, win-24540, 2α-cyano-4α,5α-epoxy-androstan-17β-ol-3-one; inhibitor of 3βHSD and 20αHSD
yr, year(s)
Δ4 (as prefix), indicating Δ4-3-keto-moiety in steroids
Δ5 (as prefix), indicating Δ5-3β-hydroxy-moiety in steroids

introduction



part I

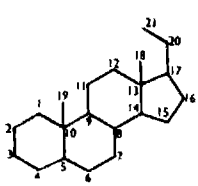
INTRODUCTION

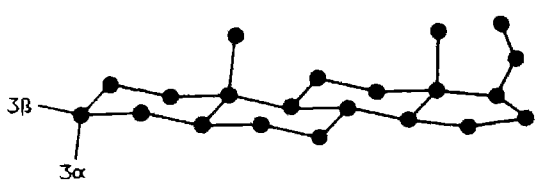
a short history

The transplantation experiments of cock testes performed by John Hunter in approximately 1771 [1] and by Arnold Berthold in 1849 [2] are now considered as the beginning of the era of endocrinology. They demonstrated that transplantation of the testes of castrated cocks between the intestines resulted in a maintainance of the normal sexual behaviour and other characteristics of cocks, that are quite different from those of capons. Berthold concluded that internal secretory products of the testes were taken up by the blood and were subsequently transferred to other parts of the body. In 1850 Franz Leydig discovered the interstitial cells [3] that were named after him, but it was only in 1903 that Ancel and Bouin [4] indicated that these cells were responsible for the development of the secondary sex characteristics. In 1889, the French physiologist Brown-Séquard reported that he had attempted self-rejuvenation, at age 72, by administering subcutaneous injections of extracts of dog and guinea-pig testes [5]. In view of the low concentrations of androgens that his aqueous extracts must have contained, this rejuvenating effect was most probably the result of auto-suggestion. He himself eventually admitted that [5]. Nevertheless, his reports stirred up a great deal of controversy and were probably largely responsible for the start of the research on the testes that followed.

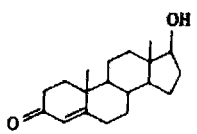
In 1935 the Dutch group of Laqueur [6] reported the isolation of a crystalline testicular compound that was highly active in the chick-

Schematic presentations of the pregnane skeleton. The hydrogens have been omitted for clarity and both the 3α- and 3β-positions are indicated.





comb test and was named testosterone (T). Although he had announced this finding already at the 'Sex Research' congress in London in 1930 [6], he hesitated to publish the results since Butenandt and Scherning in 1931 reported the isolation of 15 mg of a crystalline compound (androsterone) from 15,000 liters of human male urine [6,7] and claimed it to be 10-fold more active in the chick-comb test than the preparation of Laqueur. Later Butenandt had to admit he had been mistaken with respect to the activity [6]. The structural formula he had proposed for androsterone was proven to be correct by Ruzicka *et al* who synthesized it in 1934 from one of the stereoisomers of dihydrocholesterol [7]. Butenandt and Dannenbaum, also in 1934, isolated another compound from urine, that was later synthesized from cholesterol (Chol) and proven to be identical to DHEA [7]. Both the laboratories of Butenandt and of Ruzicka reported in 1935 that Laqueur's compound T was identical to 17ß-hydroxy-4-androsten-3-one by synthesizing it from DHEA [7]. Until the isolation of DHT in 1961 [8,9], T remained the most potent androgen synthesized so far.



testosterone

The early experiments on the biosynthesis of testicular steroids have been reviewed by Dorfman, Menon and Forchielli in 1968 [10]. Although Koch in 1937 already postulated that Chol might be the natural precursor of steroid hormones, it was only in 1953 that Ungar and Dorfman demonstrated that this hypothesis was probably correct, since they isolated ^{14}C-labeled androsterone and etiocholanolone from the urine of a patient with a virilizing adrenal tumor after administration of [3-^{14}C]Chol. In 1952 Savard, Dorfman and Poutasse first showed that P4 could be converted to T via 17OHP4 and A4, a pathway now known as the Δ4 pathway. Evidence for the existence of another pathway, the Δ5 pathway, leading from P5 via DHEA to T,was not published until 1961 [10]. Ever since the prevalence of the Δ5 or Δ4 steroid biosynthetic pathway in the synthesis of T has been demonstrated in testicular homogenates, testicular fragments, and - less often - Leydig cell cultures of many animal species [11-16]. For example, the Δ4 pathway was found in the testes of the guinea-pig, hamster, mouse and rat,

whereas the Δ5 pathway was the preferred route in the rabbit, dog and pig testes [11-16].

As far as we know, the first systematic studies on *human* testicular steroidogenesis were published in 1972 [17,18]. In these reports Yanaihara and Troen indicated that the Δ5 pathway from P5 via A5 to T was the preferred one in minced testicular fragments from prostatic carcinoma patients. They were unable to detect synthesis of 5α-reduced steroids or estrogens. The absence of measurable aromatase activity [19-23] and, with some exceptions [19,24] of 5α-reductase activity [20,21,23,25,26] in human testes was later confirmed by others.

In spite of the gradually accumulating evidence for the prevalence of the Δ5 pathway in human testes [23,25,27-29], many authors kept on studying human testicular steroidogenesis by incubating testicular fragments or homogenates with Δ4 steroidal substrates. Using [^{3}H]P4 as substrate the presence of 20αHSD [20,21,23,26,30-32] and 16αOHase [26,30,31,33] activities in human testes could be demonstrated. The low or even undetectable levels of A4 and T that were formed from P4 indicate that lyase was rate-limiting along the Δ4 pathway in human testes [20,21,23,26,29,31]. The physiological role of P4 remained controversial as the yields of P4 using radiolabeled P5 as substrate reportedly varied between <2% and almost 40% [17,22,23,26].

So far only few papers have been published dealing with testicular T synthesis by isolated human Leydig cells [34,35]. None of them, however, paid due attention to the preferential routes of steroid biosynthesis. Only Huhtaniemi *et al* [34] determined the basal and hCG-stimulated production of P5, P4, 17OHP4, A4 and T by whole testicular tissue incubation and indicated T biosynthesis via the Δ4 pathway to be similar in man and rats.

scope of this thesis

The data presented above indicate that there was still much to learn about human testicular steroidogenesis. In contrast, much was known in this respect in rats, both in testicular homogenates and in Leydig cell cultures. However, in most reports on animal and human steroid biosynthesis in testicular homogenates, metabolism of radio-labeled P5 has only been measured after a fixed incubation time, whereas dynamic time studies are scarce and do not cover the early 15

min. Experiments therefore first had to be directed to the development of a technique for the study of *in vitro* metabolism of steroids (especially [^{14}C]P5) in testicular homogenates of rats, with special emphasis on the identification and sequential measurement of the major Δ5 and Δ4 steroids by means of HPLC. Subsequently the metabolism of [^{14}C]P5 in rats was compared with that in human testicular homogenates from patients with prostatic carcinoma, hydrocele or spermatocele. The large differences between the 2 species in the conversion rates of P5 to its metabolites, in the preferred steroidogenic pathways, and in the pattern of so-called 'unidentified metabolites' formed, prompted us to study testicular steroid biosynthesis in species of animals more close to man, i.e. nonhuman primates. Large quantitative and qualitative differences in steroidogenesis were found also between humans and 2 species of macaques, not only in the preferred routes of T biosynthesis but also in the synthesis of 'unidentified metabolites'. Many experiments were performed to characterize these metabolites in the species studied. The most remarkable finding was the identification of 16-androstenes in the human testes, known (precursors of) human sex pheromones. The technique developed also enabled study of the effects of several compounds on the microsomal metabolism of [^{14}C]P5 in addition to detailed mechanistic studies of enzyme catalyzed reactions.

The study of steroid metabolism in testicular homogenates precluded observations on the mitochondrial conversion of Chol to P5 and on several receptor and 2nd messenger systems. To enable this kind of studies a technique for the isolation and culture of human (prostatic carcinoma) and rat Leydig cells had to be developed, that also enabled separate study of the mitochondrial and microsomal steroidogenic enzymes. The steroidogenic response to hCG as well as the metabolism of [^{14}C]P5 by cultured Leydig cells were studied in detail and compared with the results obtained in testicular homogenates. The data for the first time indicate that the Δ5 pathway is the preferred route in human testicular steroid biosynthesis, not only in homogenates but also in cultured Leydig cells. Furthermore these cells were found to be the major source of the 16-androstenes, (precursors of the) sex pheromones unique to pigs and human primates.

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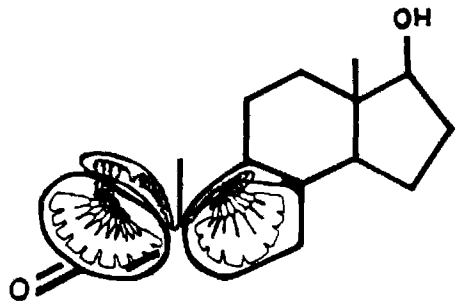
time sequence studies of the metabolism of pregnenolone in testicular homogenates



part IIa

EARLY TIME SEQUENCE IN PREGNENOLONE METABOLISM TO TESTOSTERONE IN HOMOGENATES OF HUMAN AND RAT TESTIS.

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ABSTRACT

The time sequence of the metabolism of [4-^{14}C]pregnenolone to testosterone in homogenates of human and rat testis was studied with special emphasis on the chain of events in the early 15 min of incubation. The incubations were performed at 32°C in the presence of NAD and a NADPH-generating system. The various intermediate steroids were separated by means of HPLC using a silica aliphatic diol column. Correction for procedural losses was performed by dual labeling. The present study confirms earlier reported results which showed that in the rat metabolism of pregnenolone to testosterone proceeds via the Δ4 pathway. However,this discloses for the first time that the conversion of pregnenolone proceeds very fast: progesterone, 17α-hydroxyprogesterone, and 17α-hydroxypregnenolone as the only important Δ5 intermediate, peak and decline again to almost undetectable levels within the first 15 min of incubation. Androstenedione and testosterone start to accumulate from 1 min on under the conditions used. In contrast, in the human testis homogenates, metabolism of pregnenolone to testosterone proceeds comparatively slowly and almost exclusively via the Δ5 intermediates dehydroepiandrosterone and androstenediol. Testosterone makes its appearance only after about 8 min of incubation. The data illustrate the importance of short-term incubations in evaluating the metabolism of steroids.

Endocrinology 120: 1909-1913, 1987

INTRODUCTION

It has firmly been established that in the rat testis pregnenolone (P5) is converted to testosterone (T) mainly via the Δ4 pathway, involving the intermediates progesterone (P4), 17α-hydroxyprogesterone (17OHP4), and androstenedione (A4) [1,2]. The relative importance of the intermediates of the Δ5 pathway [17α-hydroxypregnenolone (17OHP5), dehydroepiandrosterone (DHEA), and androstenediol (A5)] is, as far as we know, not acknowledged. In the human testis, the Δ5 pathway is the one preferred for the conversion of P5 to T [2-4]. In most studies dealing with this subject, the conversion of P5 via its intermediates to T has been assessed after a fixed incubation time interval of *e.g.* 2-4 h. Reports on the sequential pattern of changes of the concentrations of the steroids with increasing time are very scarce. Yanaihara and Troen [3] reported steadily increasing T concentrations from 15 - 120 min with Δ5 steroids identified as the predominant intermediates in human testis incubates. However, the time intervals chosen do not allow conclusions as to the sequence of steroid conversions in the early 15 min of incubation. In the present study the time sequence of the *in vitro* conversion of ^{14}C-labeled P5 to T was assessed by means of HPLC measurement of the eight major steroids of both pathways in homogenates of human and rat testis from as early as 1 min after the start of the incubation.

MATERIALS AND METHODS

testis tissue

Human testis tissues were obtained from patients M.P. (57 yr) and J.L. (69 yr) who underwent orchiectomy for their prostatic carcinoma and from patients with a hydrocele (A.G., 51 yr; W.V., 64 yr) or a spermatocele (G.D., 60 yr; H.L., 65 yr; G.V., 65 yr) who were biopsied at the time of surgery. Patients H.L. and G.V. underwent spinal anesthesia and the others general anesthesia. None of the patients had taken medication known to interfere with steroidogenesis. Informed consent was obtained from all of them after approval of the protocol by the Hospital Ethical Committee. The animal testes were obtained from five adult Wistar rats each weighing about 200 g. The rats were

killed by cervical dislocation; the testes were removed and used immediately or frozen at -20°C. Human whole testes or biopsy specimens were stored in liquid nitrogen immediately after dissection.

chemicals and buffers

[^{3}H]Steroids were purchased from New England Nuclear Corp. (Boston, MA) (P4, 57.0 Ci/mmol; 17OHP4, 50.0 Ci/mmol; A4, 85.0 Ci/mmol; T, 41.6 Ci/mmol; A5, 55.0 Ci/mmol) or from Amersham International (Amersham, UK) (P5, 19 Ci/mmol; 17OHP5, 12 Ci/mmol; DHEA, 60 Ci/mmol). [4-^{14}C]P5 was purchased from Amersham International (56 mCi/mmol). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from yeast, grade II, 140 U/mg, suspension in 3.2 M $(NH_4)_2SO_4$), glucose-6-phosphate (disodium salt), NADP (potassium salt, 99%) and NAD (grade I, free acid) were purchased from Boehringer Mannheim (Mannheim, West Germany). Other chemicals were purchased from several commercial suppliers.

Tissues were homogenized in 100 mM phosphate buffer, pH 7.4, containing sucrose (0.25 M) and monothioglycerol (1 mM) [5]. The assay medium was 50 mM phosphate buffer, pH 7.4. The NADPH-generating system consisted of a solution of 1 mM NADP, 10 mM glucose-6-phosphate and 0.3 U/ml glucose-6-phosphate dehydrogenase in assay buffer. Finally, the NAD containing buffer was a solution of 1 mM NAD in assay buffer.

homogenization and incubation technique

The testes were thawed, weighed, and decapsulated if necessary, sliced, homogenized in ice cold buffer (1 ml/g wet weight using a glass-glass homogenizer). By this procedure the integrity of the mitochondria is maintained [5,6] and the microsomal enzymes are preserved [7]. Then the crude homogenate was centrifuged at 4°C for 20 min at 10,000x*g*. The supernatant was diluted with assay buffer to a final volume of about 3 ml/g testis.

The substrate, [4-^{14}C]P5, dissolved in ethanol, was diluted with assay buffer to the desired concentration of about 0.1 µg (0.3 nmol)/100 µl. Then 100 µl of this solution (containing about 20,000 cpm) were pipetted into 5-ml glass tubes. The solution containing the NADPH-generating system and the solution containing NAD (400 µl each) were added. The reaction was started by adding 100 µl homogenate under

constant shaking in air at about 32°C and terminated by adding 4 ml ice-cold diethylether with shaking.

In one experiment, [7(*n*)-^{3}H]17OHP5 was used as substrate. This tracer was mixed with with nonlabeled 17OHP5 to reach a specific activity of approximately 20,000 cpm/0.1 µg. The conditions used were the same as those in the incubations with [4-^{14}C]P5.

extraction and analysis

The incubation media and the added ether were transferred rapidly to 15-ml glass tubes (containing solutions of ^{3}H-labeled recovery tracers (about 20,000 cpm/steroid.tube) in the case of incubations with [4-^{14}C]P5). The incubation tubes were rinsed twice with ether. After vigorous shaking, the tubes containing the collected ether fractions and incubation medium (and recovery tracers) were snap-frozen and the ether layers were decanted. The extraction was repeated once. The ether was evaporated to dryness in a stream of dry air.The residue was dissolved in 1 ml *n*-hexane, filtrated through a 0.45-µm filter (Schleicher & Schuell, Keene, NH, Spartan 3) and concentrated in microvials to a final volume of about 100 µl.

The HPLC system used for the separation of the metabolites was essentially one of the systems described by Schoneshöfer and Dulce [8]. The gradient profile was varied until all eight steroids of both pathways, including A5, were completely separated. The apparatus consisted of two pumps (Waters, Milford, MA, models 510 and 6000A) with autoinjector (Waters, Wisp 710B) and fraction collector (Gilson, Middleton, WI, model 202). The column was a diol-column (Merck, Hibar LiChrosorb Diol 5 µm, 25x0.4 cm). Gradient elution was run from 100% hexane to 30% isopropanol in hexane, as indicated in Fig. 1. The flow was 1.5 ml/min, the pressure about 26 atm and the void volume 3.75 ml. A volume of 90 µl was injected and the eluate was fractionated in 210 fractions (5/min, starting 8 min after injection) in scintillation vials. Four milliliters scintillation cocktail (aqualuma) were added and the vials were assayed for ^{3}H and ^{14}C in a liquid scintillation counter (Packard, Donners Grove, IL, Minaxi) employing 1-min counting time.

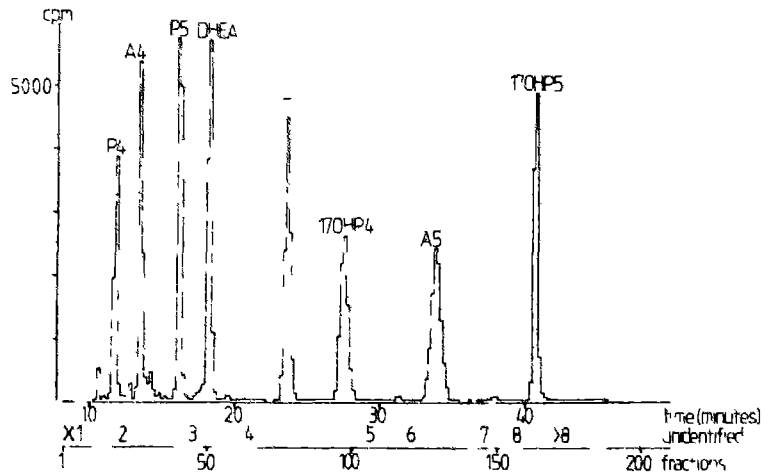

Fig. 1. Separation of the ^{3}H-labeled steroids by HPLC. Unidentified ^{14}C-labeled metabolites (X1-X8 and X>8) elute as indicated. Column, LiChrosorb Diol 5 µm, 25x0.4 cm. Solvent A, n-hexane. Solvent B, n-hexane/1-propanol 70/30 (vol/vol). Flow, 1.5 ml/min. Pressure, 26 atm. Detection, ^{3}H.

Gradient profile					
Time	*0*	*10*	*35*	*40*	*45 min*
%A:	*100*	*90*	*90*	*50*	*0*
%B:	*0*	*10*	*10*	*50*	*100*
Curve:		*linear*		*linear*	*convex*

calculations

The observed values of ^{3}H and ^{14}C were corrected for cross-over according to the standard formula.

The decision whether radioactive peaks were actually present was made according to the following criteria. Mean and SD were calculated over all the fractions of one series of incubations. The values exceeding the mean + 3xSD were considered to be statistically different from the background. The calculations were repeated, until no more significant values were detectable. The mean was used as the background for the series. Peaks in the chromatograms were defined as a number of neighboring fractions containing more counts per min than mean + 3xSD.

In order to determine recoveries, the areas under the ^{3}H-peaks were

calculated. The recovery was calculated as the quotient of the peak area and total amount of tracer added initially. The recovery of the corresponding ^{14}C-labeled metabolite was assumed to be the same.

RESULTS

HPLC steroid profile

Figure 1 shows the profile of steroid separation by HPLC. The eight major steroids could be located by their [^{3}H]markers. The reproducibility of the separation was good, with some variation in the isocratic part. The recovery of the ^{3}H-tracers varied between 45% and 65%. It has to be noted that elution of the ^{3}H- and ^{14}C-labeled steroids in the same fractions does not necessarily prove that they are identical indeed. So if a ^{14}C-labeled metabolite is named *e.g.* P4, then a steroid eluting in the same fractions as P4, probably identical to it is meant.

A number of unidentified ^{14}C-labeled metabolites (*i.e.* metabolites not corresponding to ^{3}H-markers) were detected in various eluates. They are designated X1 to X8 according to their retention times (Fig. 1). Unidentified metabolites eluting later than X8 are referred to as X>8 because their separation is poor. Of all these unidentified metabolites, only numbers 2, 5, 6, 7, and 8 are quantitatively more or less important.

time sequence of steroid profile after incubation of rat and human testis homogenates with [4-^{14}C]P5

As can be seen in Fig. 2, under the conditions of the experiment the metabolism of P5 in the rat testis homogenate proceeded rapidly via the Δ4 pathway with 17OHP5 as the only important Δ5 intermediate. All C_{21}-steroids peaked and returned to very low levels again within the first 15 min of incubation. T accumulated from 1 min on. Levels of DHEA and A5 were low throughout the series.

A number of unidentified metabolites were detected in the rat testis homogenate: X5, 6, and 8 initially after 10, 18, and 30 min, respectively. Their quantities were low (never more than 8%). A number of more polar metabolites (X>8) were detectable throughout the series.

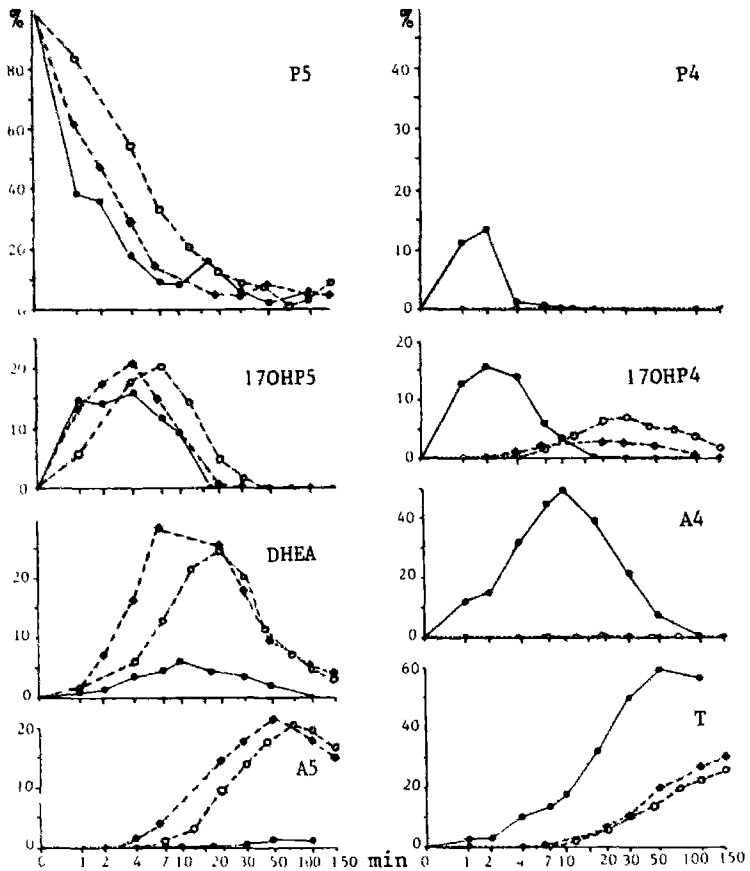

Fig. 2. Time course of the concentrations of the eight steroids of the Δ4 and Δ5 pathway in human (J.L., O– –O; M.P., ◆– –◆; prostatic carcinoma patients) and rat (●——●) testis homogenates, expressed as percentage of total amount of radioactivity added as [4-^{14}C]P5

P5 metabolism in the human testis homogenates was quite different from that in the rat and comparatively slow under the conditions used (Fig. 2 and Table 1). The Δ5 steroids 17OHP5 and DHEA were present after 1 min of incubation. Of the steroids of the Δ4 pathway, P4 and A4 were completely absent or less than 1% throughout most incubations. 17OHP4 appeared only after 4 min and accumulated slowly. From Fig. 2 it is obvious that the Δ5 pathway is the preferred one in the subjects tested, DHEA and later A5 being the major precursors of T. In contrast to the rat, T appeared only after 8 min in the human testis homogenates. Thereafter, it slowly increased to values of 26-30% after about

2 hr. After incubation of the homogenate of J.L. with 17OHP5 (*vide infra*) the results obtained (Table 2) were similar qualitatively to those obtained with P5, except for higher conversion rates to T (38%) and DHEA (45%) than when P5 was studied in the same homogenate.

In the homogenates of the testes of the spermatocele patients, levels of the Δ5 steroids were quite high and those of the Δ4 steroids quite low as compared to the other homogenates.

Table 1. Time course in minutes of the concentrations of the metabolites of P5 in human testis biopsy homogenates of hydrocele and spermatocele patients expressed as percentage of total amount of radioactivity added as [4-^{14}C]P5

Name	Time (min)	P5	17OHP5	DHEA	A5	P4	17OHP4	A4	T
Hydrocele									
A.G.	2:10	63.1	14.7	2.8	ND	ND	ND	ND	ND
	15:00	7.2	7.8	16.5	12.0	ND	9.5	ND	7.2
W.V.	1:05	73.5	8.2	1.5	ND	ND	ND	ND	ND
	8:00	13.7	19.9	19.8	2.0	1.0	3.7	0.2	0.9
	40:00	8.1	ND	11.5	15.5	ND	4.0	0.2	16.5
	150:00	7.5	ND	3.4	16.6	ND	1.2	0.2	28.3
Spermatocele									
G.D.	2:00	73.0	22.3	4.3	ND	ND	ND	ND	ND
	15:00	12.1	12.5	41.0	3.9	ND	1.5	0.8	1.1
	100:00	10.1	ND	18.6	20.8	ND	0.9	ND	14.6
H.L.	10:05	39.7	27.8	12.4	ND	ND	ND	ND	ND
	60:00	10.3	1.3	42.3	11.3	ND	ND	ND	1.6
G.V.	1:00	73.5	7.8	1.8	ND	ND	ND	ND	ND
	7:00	32.6	29.9	18.6	0.3	ND	ND	ND	ND
	30:00	9.7	1.2	52.9	4.5	ND	0.2	0.2	0.8
	150:00	7.1	ND	26.3	16.4	ND	ND	0.2	7.8

ND, Not detectable

A number of unidentified metabolites appeared in the human testis homogenates. X2 and X7 were detectable after 1-min incubation, whereas X5 appeared after 12 min. The quantities of X5 and X7 were low (less than 5%). However, X2 reached levels of 20%. A complex mixture of more polar metabolites (X>8) appeared after longer incubations. To find out whether 17OHP5 is an intermediate in the synthesis of X2 from P5, the homogenate of the testis of J.L. was incubated with [7(*n*)-^{3}H]17OHP5. In this experiment, neither X2 nor X7 were detectable at all incubation times.

In preliminary experiments with rat testis homogenates we used [4,7-^{3}H]P5 rather than [4-^{14}C]P5 as substrate. The results obtained were the same as those described in this paper. Later [4-^{14}C]P5 was used to monitor procedural losses, affording the additional advantages of an easier and more reliable peak identification and quality control of the separation via HPLC.

Table 2. Time course in minutes of the concentrations of the metabolites of 17OHP5 in the human testis homogenate of J.L. expressed as percentage of recovered radioactivity

Time (min)	17OHP5	DHEA	A5	17OHP4	A4	T
1:00	87.1	9.4	ND	1.9	ND	ND
5:00	52.1	35.2	2.2	6.6	0.5	0.9
10:00	28.6	45.0	6.8	9.5	1.2	3.2
100:00	0.6	8.8	28.2	5.2	0.3	38.1

ND, Not detectable

DISCUSSION

This study was undertaken to develop a reliable and relatively easy technique to detect enzyme deficiencies in the testes of patients suffering from different diseases.

The present study is the first emphasizing the time sequence of the conversion of P5 to androgens especially in the early 15 min of incubation in rat and human testis homogenates. It should be noted that all human samples were from older men. It can not be excluded that the pathways are different at younger ages. Furthermore it has to be stressed that the amount of [4-^{14}C]P5 added to the homogenate was far in excess of the endogenous concentration as reported by De la Torre *et al* [9], rendering interference of the results by endogenous P5 unlikely. In most studies metabolism has only been measured after a fixed incubation time, whereas time sequence studies, especially those using P5 as substrate, are very scarce and do not cover the early 15 min. From the present study it appears that in the rat 17OHP5, P4, and 17OHP4 peak within 1-2 min, whereas A4 and T make their appearance from 1 min after starting the incubation. It should be noted that except for T, which plateaus at 50 min, all major Δ4 steroids have started to decrease or nearly have returned to baseline values within

the earliest 15 min.

Although the levels of 17OHP4 and 17OHP5 were of the same magnitude the levels of A4 were much higher than those of DHEA, indicating that 17OHP4 was a much better substrate than 17OHP5 for C17,20-lyase activity. Therefore, C17,20-lyase can be considered as the rate-limiting enzyme in the Δ5 pathway in the rat testis under the conditions used. The synthesis of 17OHP4 proceeded in our experiments via two pathways, *i.e.* via P4 or via 17OHP5. This is in agreement with a study of Chubb and Ewing [1]. The ratio 17OHP5/P4 can be influenced by the concentration of the substrate P5, as P5 can inhibit 17α-hydroxylation of P4 and vice versa [10]. The relative physiological importance of both pathways remains to be determined.

A number of unidentified metabolites were detected in the rat testis homogenate. X6, appearing at about 18 min, is probably androstanediol, according to the retention time. The intermediate of the synthesis of this steroid from T, DHT, elutes very close to P5. As indicated in Fig.2, the levels of P5 rose again between 10 and 18 min, which might be attributed to the synthesis of DHT from T, although it has to be admitted that this rise only did occur at one time point. The levels of X6 were of magnitude similar to the levels of androstanediols reported by Eeckhaute *et al* [11] and Mizutani *et al* [12] using P4 as substrate.

In the human testis homogenate under the conditions used the conversion of P5 to androgens was much slower than in the rat. The predominant pathway leading to T was the Δ5 route, DHEA and A5 being the major precursors. T only appeared after about 8 min. In contrast to the rat and confirming data of Yanaihara and Troen [3], no P4 or A4 was formed throughout the time of study. The complete absence of P4 cannot be attributed to very rapid metabolism, since no Δ4 steroids were detected in the first minutes of incubation. The data suggest that under the conditions used 17α-hydroxylase is much more efficient in metabolizing P5 than 3ß-hydroxysteroid dehydrogenase, the presence of which was reflected by the appearance of 17OHP4 and T.

It should be noted that despite the virtual absence of P4, 17OHP4 slowly accumulated, suggesting that it was synthesized from 17OHP5.

Comparison of the ratios 17OHP5/DHEA and 17OHP4/A4 makes clear that in the human testis under the conditions used C17,20-lyase cleaves 17OHP5 preferably to 17OHP4. The inefficient conversion of 17OHP4 to

A4 is in agreement with a number of studies, using P4 as substrate [12,13]. However, Hosaka *et al* [14] demonstrated that C17,20-lyase can cleave 17OHP4 (confirmed by Sikka *et al* [5]), although the K_m for 17OHP4 was a factor 30 times higher than the Michaelis-Menten constant (K_m) for 17OHP5. They also demonstrated that 17OHP5 competitively inhibited the fission of 17OHP4 with an inhibition constant (K_i) of 0.6 μM. So the high levels of 17OHP5 that were detected in the first minutes of the incubation might be a cause of the lack of A4-formation.

Scrutinizing the time course changes in steroid conversion during the early 15 min it appears that DHEA precipitously increased from 1 min on whereas A5 increased only slowly. This suggests that 17-keto-steroid oxidoreductase is rate-limiting at least in the early phase. Yanaihara and Troen [3], in contrast, found a rapid accumulation of A5 from DHEA later on.

A remarkable point is the detection of relatively low levels of Δ4 steroids and high levels of Δ5 steroids in the homogenates of the patients with spermatoceles, suggesting a deficiency in 3β-hydroxy-steroid dehydrogenase enzymatic activity.

No androstanediols (having the same retention time as X6) were formed in the human testis homogenates within 150 min. This is in agreement with a number of studies using quite different conditions [12,15,16].

Remarkably, the incubation studies reveal an accumulation of hitherto unidentified metabolites appearing in either human or in rat or in both testis homogenates. The major metabolite formed in the human testis homogenates accumulating from as early as 1 min was X2 (maximum about 20%) which elutes immediately after P4 and difficultly can be separated from it. Although its structure is unknown, this compound may explain the unexpectedly high levels of P4 in some of the incubation studies reported in literature. Experiments are in progress to characterize at least some of these unidentified metabolites.

ACKNOWLEDGMENTS

The authors are indebted to the Urology Division of the St. Radboud Hospital for supplying us with the clinical material. We greatfully acknowledge Miss W. Straten for typing the manuscript.

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LIST OF TRIVIAL NAMES

androstenediol, 5-androstene-3ß,17ß-diol (Δ5)
androstanediol, 5α-androstane-3α,17ß- or -3ß,17ß-diol

ADDITIONAL COMMENTS TO CHAPTER 2

In chapter 2 the lack of synthesis of androstanediols from [^{14}C]P5 in the human testicular homogenates studied has been noted. In later experiments using testicular homogenates of numerous different patients with [^{14}C]P5 or [^{3}H]T as substrate, this lack of measurable 5α-reductase activity was invariably confirmed (see addendum B for references). However, in line with literature data [1-4], in the rat testicular homogenates [^{3}H]T and [^{14}C]P5 were converted to DHT and Aadiol, indicating that the lack of measurable 5α-reductase activity in human (and monkey, chapter 4) testicular homogenates could not be attributed to experimental shortcomings. The absence of 5α-reductase activity in human testicular tissue is rather peculiar in view of the demonstration of considerable amounts of DHT and other 5α-reduced steroids in human spermatic vein blood by several authors. In addendum B we speculate on a possible explanation of this paradox.

Aromatase activity, responsible for the conversion of the androgens A4 and T to the estrogens E1 and E2, respectively, was neither detectable in human and monkey (chapter 4), nor in rat testicular homogenates studied in this thesis, even when [^{3}H]T was used as substrate. This inability to detect aromatase activity might be related to the very low amounts of this enzyme that have been reported to be present in rat [5-7] and human [8] testicular tissues. For example, Orczyk *et al* [7] reported that the conversion of [^{3}H]T to E2 by normal rat testicular tissue was only 0.3%. The intratesticular T/E2 ratio in this species was found to be 5000 or more [6,9]. In human spermatic vein blood the reported ratios varied between 200 and 400 [10-12] and in the human testis between 70 and 100 [13-15]. To exclude the possibility that aromatase activity was not measurable in our experiments due to technical shortcomings, we studied the metabolism of [^{3}H]T in a homogenate of a normal full-term human placenta, that is known to contain a very active aromatase complex [16-19]. In this experiment, performed at 37°C, E2 was obtained in high yields (84% and 93% after 12 and 150 min, respectively). Furthermore, it has to be noted that after incubation with [^{14}C]P5 of the gonadal homogenate of a 1 month old true hermaphrodite, using standard conditions, E2 was obtained in 7% yield after 150 min, again indicating that technical shortcomings

were not the cause of the lack of measurable aromatase activity.

In chapter 2 it has been noted that testicular function changes with age. In that chapter experiments were performed using testes from elderly men, which does not exclude that the pathways of testicular steroidogenesis are different at younger age. It is, however, very hard to obtain testicular tissue from healthy, young men. In this respect it is interesting to mention the results obtained in a testicular homogenate of a 26 yr old man with cryptorchidism. In this experiment essentially the same results as in the prostatic carcinoma patients were obtained as T was synthesized from [^{14}C]P5 mainly via the Δ5 steroid A5. Also in this patient high levels of the 'unidentified metabolite' X2 were formed, while X1 and X7 were also found. Again no 5α-reduced steroids or estrogens were detected. There was no difference with respect to the preferred pathways after incubation of the homogenate of the abdominal testis at 32°C and 37°C (data not shown).

Three experiments were performed using testicular tissue from young deceased patients (road casualty, 22 yr; cerebral bleeding, 34 yr; lung cancer, 37 yr). In these experiments unexpectedly high levels of P4 were formed from [^{14}C]P5 (19-37% after 25 min), whereas the levels of the other metabolites were either low (max 7%) or virtually zero. The data indicate low activity of the cyt.P450 linked enzymes. It is well known that 17αOHase activity is highly susceptible to damage by oxygen derived free radicals [20-23], that are formed after ischemic tissue injury [23-25].

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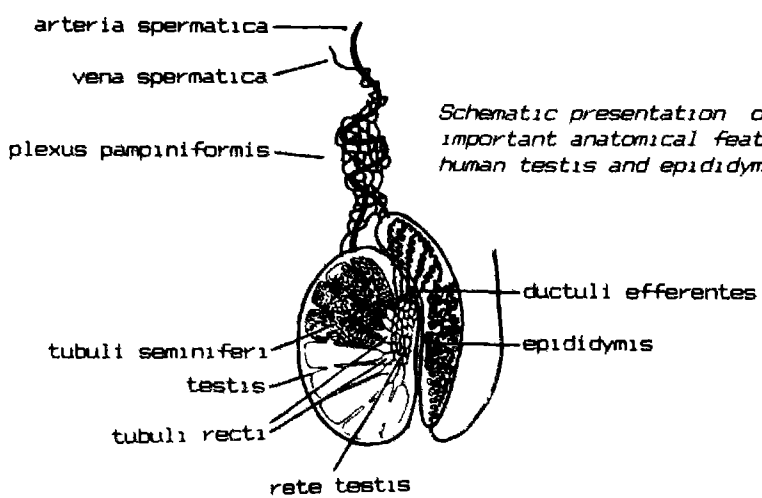
SPECULATIONS ON THE OCCURRENCE OF DIHYDROTESTOSTERONE (DHT) IN HUMAN SPERMATIC VEIN PLASMA

As already alluded to in chapter 2 and addendum A, in the human testes virtually no 5α-reductase activity is present, which is in line with literature data [1-8]. However, numerous authors showed that considerable amounts of DHT and other 5α-reduced steroids are present in human spermatic vein plasma [9-13]. Furthermore, *in vivo* administration of hCG is known to cause a rise in peripheral [10,12,14, 15] as well as spermatic vein [12] levels of DHT. In this addendum some literature data are connected with each other in an attempt to provide a possible explanation for this paradox.

The spermatozoa produced in the testis undergo further maturation in the epididymis [16-19] in an androgen (DHT) dependent process [18-23]. DHT can be synthesized in the epididymis from T since it is well-known that 5α-reductase activity is present in the epididymis [18,21,23-25]. T can reach the epididymis via the general circulation, but also via the seminiferous tubules, bound to the androgen binding protein [16,26,27]. Other authors indicate a third way, i.e. via the plexus pampiniformis [20,28]. Both the testis and the epididymal head are supplied with blood through the spermatic artery and both drain into the plexus [20,28]. A counter-current exchange of T has been shown to occur between the closely apposed artery and veins of the plexus, thus increasing arterial levels of T [20] and resulting in a



Schematic presentation of the most important anatomical features of the human testis and epididymis.

transport of T from the testis to the epididymis.

Based on these literature data the presence of high levels of DHT in human spermatic vein blood without DHT being synthesized in the testis itself can be hypothesized as follows: after its synthesis in the testis, T is excreted in the plexus pampiniformis or in the tubules. In both cases, part of the total amount of T will reach the epididymis, where it is converted to DHT. After its biological action is fullfilled, DHT is excreted by the epididymis in the veines of the plexus pampiniformis. By this theory spermatic venous DHT must be considered as an epididymal rather than a testicular steroid.

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THE SEX PHEROMONE PRECURSOR ANDROSTA-5,16-DIEN-3ß-OL IS A MAJOR EARLY METABOLITE IN *IN VITRO* PREGNENOLONE METABOLISM IN HUMAN TESTICULAR HOMOGENATES.

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ABSTRACT

In an earlier report we described the early time sequence of the *in vitro* metabolism of [4-^{14}C]pregnenolone ([4-^{14}C]P5) to testosterone in homogenates of human and rat testes and demonstrated the appearance of mainly Δ5 (humans)- and Δ4 (rats)-steroids within minutes after starting the incubation. In this study strong evidence is presented for the substantial synthesis from P5 of the sex pheromone precursor androsta-5,16-dien-3ß-ol (ADL) in human, but not rat, testicular homogenates. The 16-unsaturated C_{19} steroid ADL appeared after 1 min of incubation, and within 5 min reached values (17-23% of total radioactivity added as [4-^{14}C]P5) comparable to those of the major Δ5 steroids 17α-hydroxypregnenolone and dehydroepiandrosterone. Thus, in humans, as in boars, the sex attractant precursor ADL is a major early testicular metabolite of P5.

J Clin Endocrinol Metab 65: 753-756, 1987

INTRODUCTION

In an earlier report [1], we described the early time sequence of the *in vitro* metabolism of [4-^{14}C]pregnenolone ([4-^{14}C]P5) by homogenates of human and rat testes and demonstrated the appearance of mainly Δ5 (humans)- and Δ4 (rats)-steroids within a few minutes after starting the incubation. In addition to steroids already known, a number of unidentified ^{14}C-labeled metabolites, designated X1 to X8 and X>8, were found in human and/or rat testicular homogenates. Most of these metabolites did not reach levels of 5% of the total radioactivity added as [4-^{14}C]P5. The only exception was X2, detectable only in the human testicular incubates after 1 min and reaching levels up to 17-23% of the total radioactivity after about 5 min. In this report we present strong evidence for X2 being identical to the 16-unsaturated C_{19} steroid androsta-5,16-dien-3ß-ol (ADL), the cochromatography of ADL and the P5 metabolite in our high pressure liquid chromatography (HPLC) system, and the results of gas chromatography-mass spectrometry (GCMS) being the most important.

MATERIALS AND METHODS

A detailed description of the materials and methods concerning the homogenization, incubation and analytical techniques was reported previously [1]. In short, testis tissue was obtained from seven men, aged 51-69 yr, with prostatic carcinoma, hydrocele, or spermatocele. None had taken any medication known to interfere with steroidogenesis. Rat testes were obtained from adult Wistar rats, each weighing about 200 g. The whole testes or biopsy specimens were decapsulated, if necessary, homogenized on ice in 0.25 M sucrose buffer, and centrifuged for 20 min at 10,000×*g*. The supernatant was diluted with 50 mM phosphate buffer, pH 7.4, to about 3 ml/g testis tissue. Incubation of 100 µl homogenate with about 0.1 µg [4-^{14}C]P5 in a final volume of 1 ml was performed in air at 32°C in the presence of NAD (final concentration, 0.4 mM) and a NADPH-generating system (final concentrations, 0.4 mM NADP, 4 mM glucose-6-phosphate, and 0.12 U/ml glucose-6-phosphate dehydrogenase). The reaction was terminated by adding ice-cold diethyl ether. Tritiated marker steroids were added to monitor procedural losses. The incubation mixtures were extracted twice with diethyl

ether and analyzed by HPLC using a diol column (Hibar LiChrosorb Diol, Merck, Rahway, NJ; 5 μm) with a *n*-hexane–isopropanol gradient.

Acetylation of the steroids was performed by dissolving them in a mixture of pyridine and acetic acid anhydride (1:1, vol/vol) overnight at room temperature. The acetates were hydrolyzed by adding 4 M NaOH to a solution of the steroid acetate in the same volume of ethanol. The mixture was allowed to stand overnight at room temperature. The mixture was then neutralized by adding 2 M H_2SO_4, extracted three times with *n*-hexane and analyzed via HPLC.

To collect enough X2 for MS structure analysis, nine incubations were performed using the same conditions as described above, except that about 8 μg radioinert P5 were added to the ^{14}C-labeled P5 in each incubation as extra substrate. The fractions containing ^{14}C-labeled X2 and ^{3}H-labeled progesterone were combined and acetylated. Progesterone and the acetate of X2 were separated using the same HPLC system. The fractions containing the acetate of X2 were hydrolyzed and chromatographed again. The final yield was about 1 μg.

GCMS was performed using a Finnigan MAT 312 mass spectrometer (Finnigan Corp., Sunnyvale, CA) and a Varian 3700 gas chromatograph (Varian Associates, Palo Alto, CA). The Finnigan MAT SS300 computer system was used for the calculations. The sample was injected by an on-column injector onto a 15-m fused silica CPSIL-5CB column (Chrompack) with an internal diameter of 0.32 mm and a film of 0.11 μm. The temperature was maintained at 55°C for 1 min and subsequently raised to 300°C (5°/min). ADL eluted at about 170°C. The pressure was 1 bar, and the carrier gas used was helium. The gas chromatograph was coupled to the mass spectrometer via an open split coupling. The energy of the bombarding electrons was 70 eV, the ionizing current was 1 mA, and the temperature of the ion source was 200°C.

Tritiated ADL was synthesized by incubation with about 0.1 μg [4,7-^{3}H]P5 as substrate in the presence of the 3β-hydroxysteroid dehydrogenase inhibitor cyanoketone (100 μM) and purified by HPLC.

RESULTS

Chromatograms showing the tritiated marker steroids and ^{14}C-labeled metabolites before and after acetylation are shown in Figs. 1 and 2, respectively. As shown in these figures, X2 can be acetylated, and its

acetate is completely separated from all other metabolites. Under the conditions used, 17α-hydroxyprogesterone is not acetylatable.

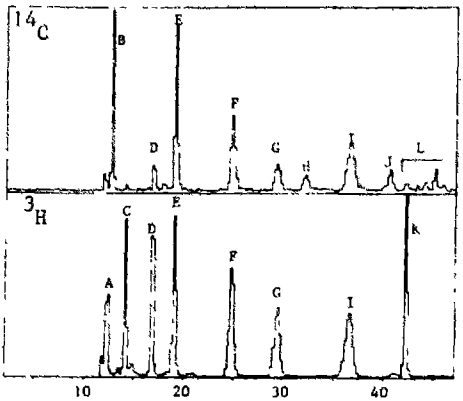

Fig. 1. A typical chromatogram showing ^{14}C-labeled metabolites of [4-^{14}C]P5 (top) and tritiated marker steroids after a 40-min testicular incubation. The homogenate used was from a 69-yr-old man with prostatic carcinoma. A, Progesterone; B, X2; C, androstenedione; D, P5; E, dehydroepiandrosterone; F, testosterone; G, 17α-hydroxyprogesterone; H, X5; I, androstenediol; J, X7; K, 17α-hydroxypregnenolone; L, X>8.

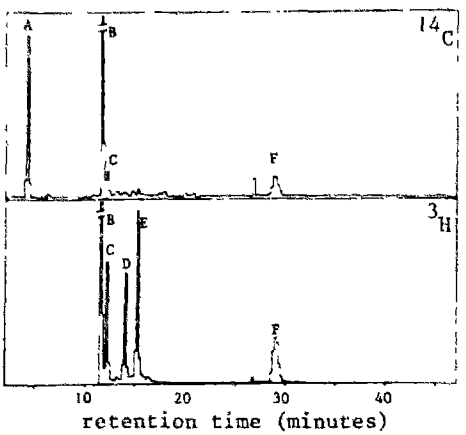

Fig. 2. Chromatogram obtained after acetylation of the marker steroids and metabolites shown in Fig. 1. A, acetate of X2; B, acetates of P5, dehydroepiandrosterone, androstenediol, and testosterone; C, progesterone; D, androstenedione; E, monoacetate of 17α-hydroxypregnenolone; F, 17α-hydroxyprogesterone.

The time sequence of formation of the major known and unidentified metabolites in the testicular homogenate of one of the prostatic carcinoma patients from our previous report [1] is shown in Fig. 3. The Δ5-steroids 17α-hydroxypregnenolone and dehydroepiandrosterone were present after 1 min of incubation and peaked after 10 and 20 min, respectively, in the testicular homogenates of all patients tested. Androstenediol and testosterone appeared only after about 4 and 8 min, respectively. Of the steroids of the Δ4-pathway, progesterone and androstenedione were absent or very low throughout the incubations, while 17α-hydroxyprogesterone seldom reached levels higher than 5% (data not shown) [1]. The unidentified metabolites X2 and X7, always

appearing together, were found after 1 min of incubation and plateaued after 5 min at levels of 17-23% and 3-4% of the total, respectively. The ratio of X2 to X7 was roughly constant during the first 30 min of incubation. In rat testicular homogenate no X2 or X7 could be detected (data not shown). X5 only appeared after about 10 min in both human and rat testicular homogenates (maximum about 5%).

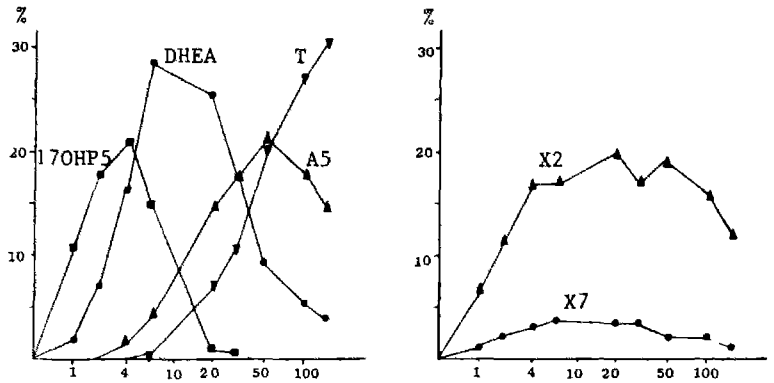

Fig. 3. Time course of the appearance of the major known and unidentified metabolites of P5 in the testicular homogenate of a 57-yr-old prostatic carcinoma patient, expressed as a percentage of the total radioactivity added as [4-^{14}C]P5. 17OHP5, 17α-Hydroxypregnenolone; DHEA, dehydroepiandrosterone; A5, androstenediol; T, testosterone.

Figure 4 presents the mass spectra of ADL and X2, providing final evidence that X2 and ADL are identical.

After incubation of a testicular homogenate with about 0.1 μg ADL, only one quantitatively important metabolite was formed (maximum about 20% of recovered radioactivity), eluting with the same retention time as the P5 metabolite X1. Detectable amounts of dehydroepiandrosterone, androstenediol, or testosterone were not found.

DISCUSSION

In this report strong evidence is presented for the hypothesis that the early unidentified metabolite X2 formed from P5 by human testicular homogenates is identical to the 16-unsaturated C_{19} steroid ADL, a sex pheromone precursor. This evidence is based on a number of facts. First, X2 and ADL eluted with the same retention time in the HPLC

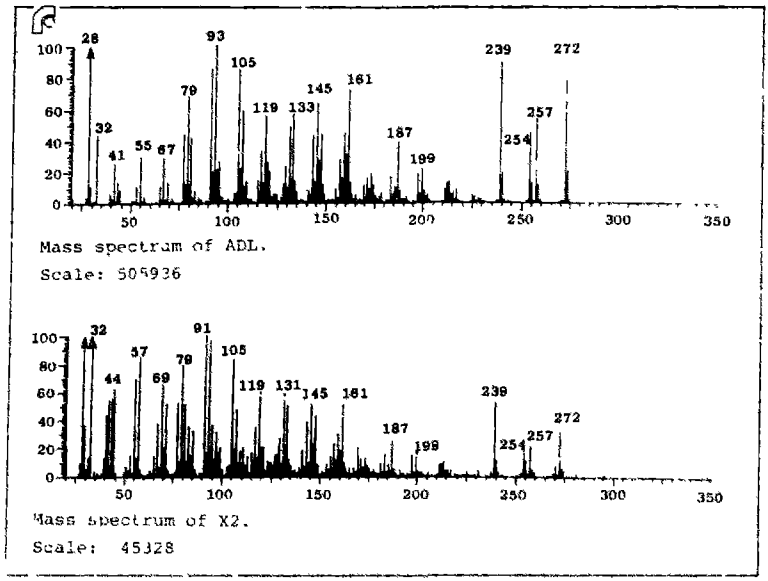

Fig. 4. The GCMS spectra of ADL and X2.

system. Second, the acetates of X2 and ADL eluted with the same retention time. As for the acetate of P5, the acetate of X2 could be hydrolyzed again to a steroid eluting with the same retention time as X2, suggesting X2 is stable under these alkaline conditions. Third, the synthesis of X2 was not blocked by cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase, suggesting X2 is a Δ5-steroid (our unpublished data). Fourth, the compound SU-10603, which is known to inhibit several cytochrome P450-linked oxidations, strongly inhibited the synthesis of X2 (our unpublished data). As reported by Nakajin *et al* [2], the conversion of P5 to ADL was inhibited by this compound in boar testis. Fifth, the mass spectra of X2 and ADL were identical.

Data on ADL in human testes was scarce. In 1971 Bicknell and Gower [3] demonstrated that the testes were a source of ADL, since their removal resulted in a dramatic decrease in its urinary level in two patients with testicular feminization. Furthermore, in the same patients these researchers provided some evidence that ADL was formed from P5 in testes *in vitro*, with percent yield of 1-5%. We now demonstrate that even before testosterone can be detected, quantitatively significant amounts of ADL, comparable to those of the major Δ5-steroids, are synthesized from P5 by homogenates of testes from patients with no endocrine disorders. In 1973 Ruokonen [4] demonstrated the

presence of free and sulfate-conjugated ADL in cadaver testis tissue.

The concept of X2 being ADL raises some interesting possibilities. The enzyme 3β-hydroxysteroid dehydrogenase can convert ADL to androsta-4,16-dien-3-one (ADN). Metabolite X1, the only quantitatively important metabolite formed after incubation with ADL and detectable in some incubations with P5, may be identical to ADN. Furthermore, this highly nonpolar steroid can be metabolized peripherically to the odorous steroids androstenone (urine smelling) and androstenol (musk smelling) [5] by the same types of enzymes that convert testosterone to androstanediol. These 16-unsaturated C_{19}-steroids have been found in human urine [5,6], blood [6], saliva [7], and axillary sweat [6,8] and may serve as human sex pheromones [9,10], as is the case in pigs [5].

Most of the studies on ADL and related compounds, apart from the scarce data in human testes, dealt with boar testes, where large amounts of 16-unsaturated C_{19}-steroids were detected [5,11,12]. The rate of formation of ADL from P5 described in this paper in men was similar to that in boars, which also rapidly synthesized substantial amounts of ADL [5]. In boar testes, 21-hydroxypregnenolone was proposed to be an intermediate in the synthesis of ADL from P5 [13,14]. In the human testis, however, this seems unlikely,since in our experiments ADL was detected within the first minute of incubation, whereas no metabolite eluting in the region of 21-hydroxypregnenolone (*i.e.* between 17α-hydroxyprogesterone and androstenediol) was detected.

In rats, data on ADL are lacking, but the *in vitro* conversion of P5 to ADN and 5β-androst-16-en-3α-ol was very low [5]. Our failure to find measurable quantities of ADL in rat testicular homogenates in is line with this finding.

Shimizu and Nakada reported the synthesis of ADL [15,16] and androst-5-ene-3β,17α-diol [16,17] from P5 by boar testicular microsomes in the presence of a NADPH-generating system and in the absence of NAD. In our view, X7 is identical to androst-5-ene-3β,17α-diol. This idea is supported by our findings that the synthesis of X7, like that of X2, was not blocked by cyanoketone, but was strongly inhibited by SU-10603 (see above). We have some evidence for the hypothesis that X1 and ADN are identical.

In conclusion, in man, as in boars, the 16-unsaturated C_{19} steroid ADL is a major early metabolite of *in vitro* testicular P5 metabolism.

ADL may act as a precursor to the odorous sex attractant steroids androstenone and androstenol, which may be important in human sexual relations.

ACKNOWLEDGMENTS

The authors are indebted to J.B. Huesem, G.J.H. Schmeits, and P.L. Jacobs [AKZO Research Laboratory (Arnhem, The Netherlands) and Organon International (Oss, The Netherlands)] for making the mass spectra.

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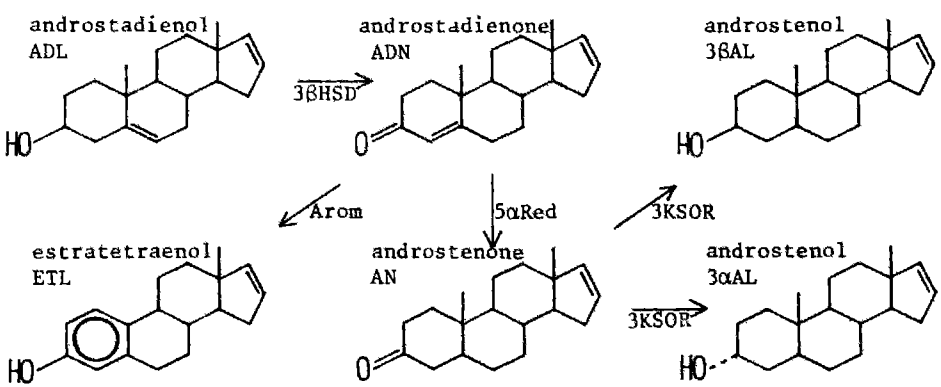
LIST OF TRIVIAL NAMES

androstenediol, androst-5-ene-3ß,17ß-diol
androstenol, 5α-androst-16-en-3ß- or -3α-ol
androstenone, 5α-androst-16-en-3-one
cyanoketone, 2α-cyano-17ß-hydroxy-4,4,17-trimethylandrost-5-en-3-one
pregnenolone (P5), 3ß-hydroxypregn-5-en-20-one
SU-10603, 7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)naphtalenone

ADDITIONAL DATA CONCERNING THE OCCURRENCE AND PHYSIOLOGICAL ROLE OF 16-ANDROSTENES

In chapter 3 the identification of metabolite X2 as ADL has been described and some reference was made to the physiological properties of the 16-androstenes. As already mentioned in this chapter, literature data on ADL and other 16-unsaturated steroids in humans are scarce. In 1950 and 1952 Brooksbank and Haslewood isolated AL from the glucuronide fraction of human male [1] and female [2] urine, respectively. Later several 16-unsaturated C_{19} steroids have been found to be present in human urine [3-6], plasma [7-11], axillary sweat [11-17], fat [11], and saliva [18]. In all these studies the reported concentrations were much higher in men than in women. The phenolic $\Delta 16\text{-}C_{18}$ steroid estratetraenol has been found in urine of pregnant women [19]. In 1971 Bicknell and Gower [3] indicated the testes as a source of ADL since their removal resulted in a dramatic decrease in its urinary level in 2 patients with testicular feminization. Furthermore these authors provided some evidence that ADL was formed from P5 *in vitro* with yields of 1 to 5% in the same patients. In 1973 Ruokonen [20] demonstrated the presence of free and sulfate conjugated ADL in human cadaver testes.

Most studies on 16-unsaturated C_{19} steroids dealt with the boar testis, where large amounts of these compounds were detected [21]. In 1944 Prelog and Ruzicka [22] first isolated 35.4 mg 3αAL and 39.5 mg 3βAL from 181 kg boar testes. They were unable to correct their yields



for procedural losses but it is now generally accepted that in porcine testis tissue the 16-androstenes, in both free and sulfated form, are quantitatively more important than other steroids, including T and its precursors [21,23-28]. Numerous 16-androstenes were found to be present in boar fat [29-34], submaxillary glands [23,34-36], saliva [21,32], plasma [9,37-39], urine [21,39], and sweat [40].

An interesting feature of the 16-androstenes is their intense odour. The musk-like odour of AL [1,2,22,34,41,42] and the urine-like or perspiration-like odour of ADN and AN [18,30,42,43] are well-known and amongst others responsible for the 'boar taint' that can be detected in meat of mature, uncastrated male pigs [30,31,34,43]. Prelog *et al* [22,42] and Brooksbank and Haslewood [1,2] were able to detect the odour of AL in different fractions obtained during its isolation. In a pilot study in our laboratory 53 out of 58 healthy volunteers aged 20 to 53 yr were able to detect the odour of ADL and although no difference in ability to detect the odour was found between the sexes, most male volunteers used positive terms to describe the odour (such as "soap, aftershave, peppermint, sweet"), whereas most female volunteers detested the odour ("terrible, urine, sweat, hospital-like") [16,30,44]. These odorous characteristics are probably related to the relatively low melting points of these steroids (120 to 140°C *versus* 200 to 300°C for most other naturally occurring steroids [45]) and are probably important in respect to the sex pheromone properties of these compounds.

Numerous studies indicate that the 16-androstenes, especially the 5α-reduced steroids AN and AL, act as sex pheromones in pigs [21,26, 32,34,36] and evidence has been provided that these steroids have similar functions in humans [47-52] and act stimulatory in women. They lack androgenic activity [21]. The data presented in this thesis indicate that ADL is secreted by the human testis without substantial further metabolism in this gland since its metabolism in testicular homogenates was restricted to formation of some ADN. Due to the virtual absence of 5αRed activity, synthesis of AN or AL was not expected but other possible metabolites as 16,17-epoxydes or -glycols [53-56] were not found either. AN and AL can be synthesized from ADL via ADN peripherically, in tissues that excrete these steroids, or by skin microorganisms [13,17]. The presence of 3βHSD and 5αRed activity in human skin [57-62], especially in the axillary sweat glands, and

(preliminary) evidence for 5αRed activity in human salivary glands [63,64], gingival tissue [65] and lungs [66] have been reported in literature.

Although large amounts of ADL (and to a lesser extent ADN (X1)) were formed from [^{14}C]P5 in human testes, they were not found in the testicular homogenates of the monkeys tested (see chapter 4). So far, the presence of 16-androstenes seems to be limited to men and boars [11,21,49]. The genuine musk, coming from the male musk-deer, is not a steroid but a macrocyclic ketone (3-methylcyclopentadecanone) [22,42, 46,51].

Finally, another comment has to be made. As already referred to earlier, the substantial synthesis of the sex pheromone precursors ADL and ADN in the testis indicate that testicular steroidogenesis, apart from the well-known functions, is also actively involved in the process of sex-attraction. Nowadays, however, this role is possibly overruled by modern 'exciting and irresistable' artificial odours of aftershaves, eau de toilettes and so on. It has to be noted that many of these cosmetics are an odorous composition of a variety of flowers with a heart of musk.

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CHEMICAL SYNTHESIS OF [^{3}H]ADL

Since [^{3}H]ADL was not commercially available, experiments were performed to synthesize this steroid. Initially, this goal was achieved by incubating a human testicular homogenate with [4,7-^{3}H]P5 as described in chapter 3. Later [^{3}H]ADL was synthesized according to the method described by Watabe *et al* [1]. [1,2,6,7-^{3}H(N)]DHEA (100 µCi, 97.3 Ci/mmol) (New England Nuclear Corp, Boston, MA) was dissolved in methanol (1.5 ml) containing *p*-toluenesulfonylhydrazine (0.66 mg) (Janssen Chimica, Beerse, Belgium) and heated for about 5 hours under reflux conditions in a water bath of 70-75°C. After evaporation of the solvent from the mixture in a nitrogen stream, the resulting residue was dissolved in chloroform (5 ml) and washed successively with 2 M HCl (2.5 ml), 3% $NaHCO_3$ (1 ml) and water (1 ml). A residue obtained on the evaporation of the solvent from the solution was dried over phosphorus pentoxide overnight *in vacuo* and dissolved in anhydrous tetrahydrofuran (100 µl). To this solution covered with nitrogen a solution of n-butyllithium in anhydrous n-hexane (30 µl, 1.6 M) (Janssen Chimica) was added, and the mixture was kept overnight at room temperature. The mixture was then diluted with an admixture (5 ml) of toluene and methanol (10:1) and washed successively with 5% H_2SO_4, 3% $NaHCO_3$ and water (1 ml each). A residue obtained on the evaporation of the solvent from the washed organic solution in a nitrogen stream contained ADL in a radiochemical purity of about 40%. The compound was purified via HPLC (see chapters 2 and 4).

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DIFFERENTIAL METABOLISM OF PREGNENOLONE BY TESTICULAR HOMOGENATES OF HUMANS AND TWO SPECIES OF MACAQUES. LACK OF SYNTHESIS OF THE HUMAN SEX PHEROMONE PRECURSOR 5,16-ANDROSTADIEN-3ß-OL IN THE NONHUMAN PRIMATES.

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ABSTRACT

In previous reports we described the early time sequence in *in vitro* [4-^{14}C]pregnenolone metabolism in human and rat testicular homogenates and, apart from a difference in the preferred route of the conversion of pregnenolone to testosterone, we demonstrated the presence of 16-ene-synthetase activity in human but not in rat testes.

In the study of testicular function higher monkeys are increasingly used as a model for human reproduction. The availability of testes from 2 different species of macaques (2 crab eating monkeys and 1 rhesus monkey) enabled us to compare the *in vitro* metabolism of pregnenolone in these testes with human testes. The patterns obtained in both monkey species were very similar but completely different from those found in man. The Δ4 pathway was the preferred route for the conversion of pregnenolone to testosterone in the monkeys tested, the Δ5 pathway in the humans. In the monkeys substantial amounts of 20α-dihydroprogesterone and 17α,20α-dihydroxy-4-pregnen-3-one were formed from [^{14}C]pregnenolone whereas in man the latter compound was detectable only using [^{3}H]progesterone as substrate. 16-Ene-synthetase activity, a prerequisite for the synthesis of the sex pheromone precursors 5,16-androstadien-3ß-ol and 4,16-androstadien-3-one, was clearly measurable in the human but not in the monkey testicular homogenates. Evidence for the presence of 16α-hydroxylase, an enzyme reported to be unique to human and subhuman primates, was found in both, making man the only primate studied so far coharbouring in its testes both 16-ene-synthetase and 16α-hydroxylase activities. These *in vitro* data indicate that the nonhuman primates studied are not suitable models for the study of human testicular function.

J Steroid Biochem. submitted

INTRODUCTION

In previous reports we described the early time sequence of the metabolism of [4-^{14}C]pregnenolone (P5) in homogenates of human and rat testes [1] and we demonstrated the substantial synthesis of the 16-unsaturated C_{19} steroid 5,16-androstadien-3β-ol (ADL), a sex pheromone precursor, by human but not by rat testis homogenates [2]. Apart from the presence or absence of 16-ene-synthetase activity in human and rat testis homogenates a number of other differences were found between these species, such as the preferred pathways leading from P5 to testosterone (T) (Δ5 route in man, Δ4 route in rat) and the presence or absence of 5α-reductase activity. In the past, rodents have long been utilized in the study of testicular function, but nowadays monkeys are increasingly used as a model of human reproduction [3]. The availability of testes from 2 species of higher monkeys (rhesus and crab eating monkeys) enabled us to compare the early time sequence in P5 metabolism in testicular homogenates of human and subhuman primates. Special attention was paid to the preferential pathway of T biosynthesis and the presence of 16-ene-synthetase in addition to 16α-hydroxylase activity. The latter has been reported to be unique to human and subhuman primates [4].

MATERIALS AND METHODS

chemicals

[^{3}H]Steroids were purchased from New England Nuclear Corp. (Boston, MA) or from Amersham International (Amersham, UK). [4-^{14}C]P5 was purchased from Amersham International (56 mCi/mmol). Other chemicals were purchased from several commercial suppliers.

testis tissue

The testes of 3 adult monkeys (1 rhesus monkey, *Macaca mulatta*, and 2 crab eating monkeys, *Macaca irus*) were dissected within 1 hr after sacrifice in october, april and august, respectively. The human testes were obtained from 3 patients (57-73 yr) who underwent orchidectomy for their prostatic carcinoma. The data of 2 of them were reported in

earlier papers [1,2]. None of the patients or macaques received medication known to interfere with steroidogenesis. The testes were stored at -80°C.

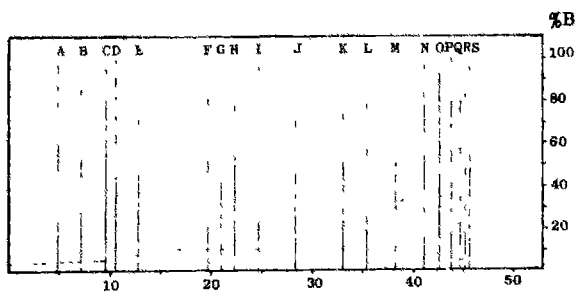
techniques of tissue homogenization, incubation, and analysis of the metabolites

The techniques of homogenization, incubation and analysis had been described in detail elsewhere [1]. In short, testes were weighed, decapsulated if necessary, homogenized on ice in 100 mM phosphate buffer pH 7.4 containing 0.25 M sucrose and centrifuged at 10^4xg (20 min, 4°C). The supernatant was diluted with 50 mM phosphate buffer pH 7.4 to a final volume of about 3 ml/gram wet weight. Unless indicated otherwise, 100 µl homogenate was incubated in a final volume of 1 ml with the radiolabelled steroid indicated (usually [4-^{14}C]P5) in the presence of NAD (final concentration, 0.4 mM) and a NADPH generating system (final concentrations, 0.4 mM NADP, 4 mM glucose-6-phosphate, 0.12 U/ml glucose-6-phosphate dehydrogenase) in air in a shaking water bath at 32°C. The reaction was terminated by adding ice cold diethylether. In the cases that a carbon labelled substrate was used, ^{3}H marker steroids (P4, A4, P5, DHEA, T, 17OHP4, A5, 17OHP5, and oestradiol) were added to monitor procedural losses. After double extraction with ether the mixture was analyzed by HPLC using a diol-column (Hibar LiChrosorb diol, Merck, Rahway, NJ; 5 µm) with a n-hexane/isopropanol gradient as indicated in fig 1. The eluate was fractionated in 154 scintillation vials (3 vials/min) and assayed for ^{3}H and ^{14}C in a liquid scintillation counter. In the cases that [^{3}H]substrate was used, yields are expressed as percentage of total recovered radioactivity. In the cases that [^{14}C]substrate was used, the recoveries of the ^{3}H markers were calculated as the quotient of the peak area and the amount of tracer added initially. The recoveries of the corresponding ^{14}C labelled metabolites were assumed to be the same. The recoveries of ^{14}C labelled metabolites not identified by ^{3}H markers were estimated by interpolation between the recoveries of both neighbouring markers. Yields are expressed as percentage of total radioactivity added as ^{14}C labelled substrate.

remarks

Although HPLC is a powerful tool to separate a great number of steroids (see fig 1), it has to be noted that when 2 steroids elute with the same retention time, they are not necessarily identical indeed, although it has to be noted that very small differences in retention times are measurable in each run due to the use of ^{3}H markers and the fractionation in 154 vials. So if a metabolite is named for example A5, then *a metabolite eluting in the same fractions as A5 and probably identical to it* is meant.

Fig 1. Chromatographic separation of the steroids. The dotted line represents the gradient profile. Solvent A, n-hexane. Solvent B, n-hexane/isopropanol 70/30 (vol/vol). Flow, 1.5 ml/min. Pressure, 26 atm.

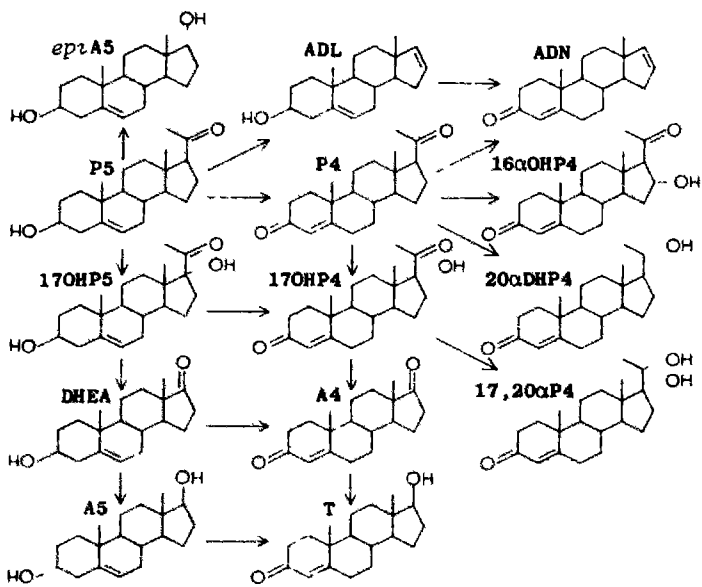


A, ADN; B, 5α-androstane-3,17-dione; C, P4; D, ADL; E, A4; F, P5; G, 17β-hydroxy-5α-androstan-3-one; H, DHEA; I, 20αDHP4; J, T; K, 17OHP4; L, X5; M, 5α-androstane-3α,17β- and -3β,17β-diol; N, A5; O, epiA5; P, 17OHP5; Q, 17,20αP4; R, oestradiol; S, 16αOHP4.

A number of 'unidentified metabolites', i.e. metabolites not identified by ^{3}H-markers, were formed from [^{14}C]P5 in the testis homogenates of the rats [1], monkeys and men. They were designated X1 to X10 according to their retention times. They were tentatively identified as ADN (X1), 20αDHP4 (X4), *epi*A5 (X7), 17,20αP4 (X9) and 16αOHP4 (X10). In short, the putative precursors were selected by incubations of testicular homogenates with [^{14}C]P5 or ^{3}H-labelled P4, 17OHP5, 17OHP4, DHEA, A4, A5, T or ADL. The enzymatic activities responsible for the synthesis of the metabolites were partially characterized by studying the effects of the inhibitors epostane [5] and SU-10603 [6], and the presence or absence of cofactors (NAD,

NADPH) [7]. Comparison of the profiles of products that were formed when the metabolites were acetylated (25 µl pyridin, 25 µl acetic acid anhydride) using different reaction times (1-150 min, reaction stopped by adding 0.5 ml ethanol, analyzed via HPLC) provided information as to the number of acetylatable hydroxylgroups present. Finally, the retention times of authentic standards were compared with those of the metabolites. The nature of X5, formed from P5 in yields <5% in the testicular homogenates of rats, monkeys and men, remains unknown.

Fig 2. Biosynthesis of testicular steroids.



RESULTS

The time sequence of the metabolism of [^{14}C]P5 is presented in fig 3. The profiles obtained in the testes of the 2 species of monkeys tested were qualitatively very similar, but completely different from those found in the human testis homogenate, which are identical to the profiles we described earlier [1,2]. The synthesis of T in the human testes proceeded almost exclusively via the Δ5 steroids 17OHP5, DHEA

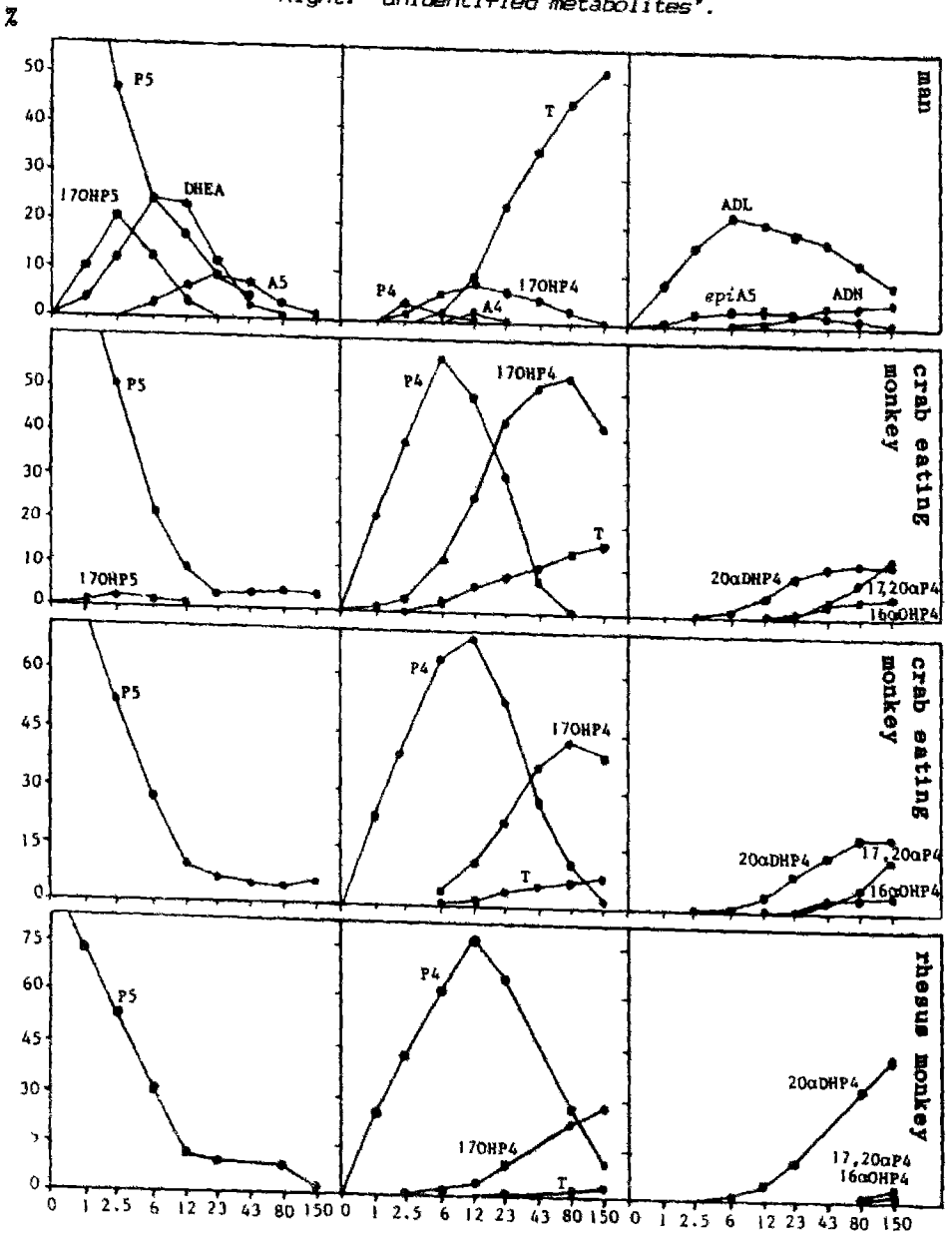
and A5. The only quantitatively important Δ4 intermediate formed was 17OHP4. The conversions of P5 to DHEA (via 17OHP5) and of A5 to T proceeded fast and substantial amounts of T were synthesized from 6 min on.

In contrast to men, the synthesis of T from P5 in the monkey testis homogenates tested proceeded exclusively via the Δ4 steroid P4; levels of 17OHP5 and other Δ5 steroids were very low or even undetectable throughout the incubations. P4 accumulated rapidly (>20% within 1 min) and peak levels (up to 78%) were achieved within 6 to 12 minutes. It was metabolized relatively slowly to 17OHP4 (reaching levels up to 54% after 80 min) which in turn was converted slowly. Levels of A4 were in most cases undetectably low. The conversion of A4 to T proceeded fast, since when [^{3}H]A4 was used as substrate in the testis homogenate of a crab eating monkey more than 50% was converted into T within 1 min. Relatively small amounts of T (max 15%) were formed from [^{14}C]P5 during the 150 min incubation.

Apart from the steroids of the Δ4 and Δ5 pathway a number of other metabolites were formed from [^{14}C]P5 under the conditions used. The profiles of these metabolites in the monkey testes were very similar but completely different from those found in the human testes (see fig 3). In the human testes large amounts (max 23%) of the 16-unsaturated C_{19} steroid ADL were formed within minutes after starting the incubation, appearing together with its satellite *epi*A5 (max 4%) [8]. ADL was converted slowly into ADN (max 5%). In the monkey testes these steroids were not formed regardless the substrate used. The quantitatively most important 'unidentified metabolites' formed in these testes were 20αOHP4 (up to 43% after 150 min), 17,20αP4 (up to 14%) and 16αOHP4 (up to 5%). 20αOHP4 was not formed in detectable amounts from [^{14}C]P5 in the human testis homogenates tested. In contrast to the monkeys, a complex mixture of low-yield, poorly separated, polar metabolites (X>8) was formed in the human testis homogenates tested after longer incubation times. Quantitatively important amounts of 17,20αP4 and 16αOHP4 were not detectable in this mixture. However, when [^{3}H]P4 was used as substrate in human testis homogenates, synthesis of 16αOHP4 and 17,20αP4 (10% and 2% after 100 min, respectively) was clearly measurable.

No detectable amounts of 5α-steroids or estrogens were formed in any of the incubations, including those using [^{3}H]T as substrate.

Fig 3. Time sequence of the metabolism of [4-^{14}C]P5. Yields are expressed as percentage of total radioactivity added as substrate ([^{14}C]P5). Metabolites with peak levels <1% are not shown. Left: steroids of the Δ5 pathway. Middle: steroids of the Δ4 pathway. Right: 'unidentified metabolites'.



DISCUSSION

This paper compares the early time sequence in [^{14}C]P5 metabolism in testis homogenates of human and subhuman primates, i.e., the rhesus monkey (*Macaca mulatta*) and the crab eating monkey (*Macaca irus*), two species of the old world monkeys close to man.

Under the conditions of the experiment the conversion of P5 in the human and monkey testicular homogenates were completely different, whereas in both monkey species the similarity in P5 metabolism was striking. In the human testis T was rapidly synthesized from P5 predominantly via the Δ5 pathway, whereas in both monkey species the Δ4 biosynthetic pathway was the preferred route, P4 peaking within a few minutes but T accumulation being relatively slow. The preference of the Δ5 pathway in human testes has been described previously [1,7,9] but there is still discrepancy in literature concerning the preferred pathway of P5 metabolism to T in monkey testes. In the more primitive primates as the marmoset P5 is converted to T exclusively via the Δ4 pathway [10,11], but in the baboon and the rhesus monkey conversion via the Δ4 as well as the Δ5 pathway has been reported [12,13]. Evidence for a preferred Δ4 pathway in the crab eating macaque has been presented by Inano *et al* [4]. Our inability to detect 5α-reductase and aromatase activity in human and monkey testes is in line with literature data [9,14,15].

In the monkey but not in the human testis homogenates large amounts of the 20α-hydroxylated steroids 20αOHP4 and 17,20αP4 and, to a lesser extent, 16αOHP4 accumulated using [^{14}C]P5 as substrate. Using [^{3}H]P4 as substrate also in men the presence of 20α-hydroxysteroid dehydrogenase and 16α-hydroxylase enzyme activities could be demonstrated, confirming data published by others [9,15-17]. The presence of 20α-hydroxysteroid dehydrogenase activity in baboon and orang utan testes has been demonstrated as well [18]. Detectable amounts of the 20β-hydroxylated steroids 20β-hydroxy-4-pregnen-3-one or 17α,20β-dihydroxy-4-pregnen-3-one were not formed in any of the incubations described in this paper. Interestingly, the latter compound acts as a sex pheromone in the goldfish, synchronizing milt production and ovulation [19].

The presence of an active 16-ene-synthetase enzyme in all human testis homogenates we studied so far and its complete absence in both

species of monkeys is rather remarkable in view of the supposedly close relation of these primates to humans, and their common capacity of the 16α-hydroxylation of P4 which is - according to Inano *et al* [4] - a characteristic of testicular function of primates. According to these authors in no other mammal (rat, mouse, guinea pig, cat, dog, goat or horse) 16α-hydroxylase has been demonstrated. The presence of 16-ene-synthetase activity is a well-known characteristic of boar testes [2,6,20,21]. Interestingly, in this species also evidence for the presence of testicular 16α-hydroxylase activity has been found [20,21] as 16αOHP5 accumulated after incubation of P5 in microsomal testis fraction in the absence of NAD. Although the presence of this enzyme in human testes has earlier been reported [9,15,17], it has received little attention. The steroid serving as substrate for this enzyme in human testes *in vivo* remains unclear since under the conditions of our experiments only small or undetectable amounts of P4 were formed from P5. 16α-Hydroxylation of P5 was not observed in any of the human testis homogenates we studied so far. Moreover substrate preference of testicular 16α-hydroxylation in man is limited to P4 [17].

In contrast to the cytochrome P450-enzymes the activity of 17-ketosteroid oxidoreductase in the monkey testes tested appeared to be very high under the conditions used. Despite the preferred Δ4 route levels of A4 formed from [^{14}C]P5, [^{3}H]P4 or [^{3}H]17OHP4 were in almost every experiment undetectably low. When no NADPH (essential for 17-ketosteroid oxidoreductase activity [7]) was added to the incubation medium no T was formed from [^{14}C]P5 and synthesis of A4 was clearly measurable. The very fast synthesis of T when [^{3}H]A4 was used as substrate (>50% within 1 min) is in line with such a high activity. The quasi absence of significant amounts of 17OHP5 in monkey and of P4 in human testis homogenates must be explained by the virtual lack of synthesis of these steroids since no fast metabolism was found using [^{3}H]17OHP5 or [^{3}H]P4 as substrate and no metabolites of these steroids were found after 1 min of incubation using [^{14}C]P5 as substrate.

In conclusion, under the conditions adopted, P5 metabolism in the testis homogenates of the subhuman primates studied differs from that in humans in many ways, the most important being the preferential use of the Δ4 pathway in the synthesis of T in monkeys against the Δ5 pathway in men and the complete lack of 16-ene-synthetase activity in

the monkey testes. This enzyme activity is a prerequisite for the synthesis of the 16-unsaturated steroids ADL and ADN, sex pheromone precursors at least in men and boars. Although 16α-hydroxylase and 20α-hydroxysteroid dehydrogenase activities are present in human as well as in monkey testes only in the latter the huge P4 synthesis allows accumulation of the 16α- and 20α-hydroxylated metabolites. This paper demonstrates that as long as his closest living relatives (chimpanzee, gorilla and orang utan) have not been studied, man is the only primate coharbouring in its testes 16-ene-synthetase and 16α-hydroxylase activities. It is concluded that the primates studied are not suitable models for the study of human testicular steroidogenesis.

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LIST OF ABBREVIATIONS

A4, androstenedione, 4-androstene-3,17-dione
A5, androstenediol, 5-androstene-3β,17β-diol
ADL, 5,16-androstadien-3β-ol
ADN, 4,16-androstadien-3-one
DHEA, dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one
20αDHP4, 20α-dihydroprogesterone, 20α-hydroxy-4-pregnen-3-one
*epi*A5, epiandrostenediol, 5-androstene-3β,17α-diol
16αOHP4, 16α-hydroxyprogesterone, 16α-hydroxy-4-pregnene-3,20-dione
16αOHP5, 16α-hydroxypregnenolone, 3β,16α-dihydroxy-5-pregnen-20-one
17OHP4, 17-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione
17OHP5, 17-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one
P4, progesterone, 4-pregnene-3,20-dione
P5, pregnenolone, 3β-hydroxy-5-pregnen-20-one
17,20αP4, 17α,20α-dihydroxy-4-pregnen-3-one
T, testosterone, 17β-hydroxy-4-androsten-3-one

CHARACTERIZATION OF 'UNIDENTIFIED METABOLITES' FORMED FROM [^{14}C]P5 IN TESTICULAR HOMOGENATES

In the previous chapters the synthesis of numerous 'unidentified metabolites' from [^{14}C]P5 in testicular homogenates from rats, monkeys and men has been stressed. They were designated X1 to X10 according their retention times in the HPLC system. The characterization of most of these 'unidentified metabolites' has been described elsewhere in this thesis (in detail in chapter 3 and in short in chapters 2, 3, 4, and 5). In this addendum the experiments leading to the characterization of these metabolites are summarized.

MATERIALS AND METHODS

The homogenization, incubation and analytical techniques are described extensively elsewhere in this thesis. It has to be noted that the HPLC gradient profile as presented in chapter 2 was later changed to optimalize the separation between P4 and X2 and between P5 and DHT, resulting in the profile presented in chapter 4.

SU, a compound known to inhibit several cyt.P450 linked oxidations [1-3], was kindly donated by C.A. Brownly Jr (Ciba Geigy, Summit, New Jersey). EPOS and TRIL, compounds known to inhibit both 3βHSD and 20αHSD [4-8], were kindly donated by D.O. Sanders (Sterling Research Group Europe, Sterling Winthrop, Haarlem, The Netherlands). Other chemicals were purchased from several commercial suppliers as described elsewhere in this thesis.

Steroids were acetylated by adding 25 µl acetic acid anhydride to 25 µl of a solution of the radiolabeled steroid in pyridine at room temperature. The reaction was terminated by adding 0.5 ml ethanol. The solvents were evaporated to dryness, the residue dissolved in n-hexane and analyzed via HPLC. In this way most primary and secundary hydroxylgroups are esterified whereas tertiary hydroxyl groups remain unesterified [9]. Comparison of the profile of products formed after 1, 5 and 100 min allows conclusions as to the number of acetylatable hydroxylgroups present.

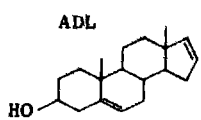
Reduction of steroids was performed by dissolving them in 1 ml water with about 10 mg $NaBH_4$ for 2 hr at room temperature. The mixture

was extracted with ether and analyzed via HPLC.

[^{3}H]ADL and [^{3}H]X7 were synthesized by incubation of a human testis homogenate with [4,7-^{3}H]P5 in the presence of 100 µM EPOS. [^{3}H]X1 was synthesized from [^{3}H]ADL in the same homogenate without addition of inhibitors. [^{14}C]20αDHP4 (X4) was synthesized by incubation of the testis homogenate of the rhesus monkey with [4-^{14}C]P5 in the presence of 100 µM SU. The testis homogenate of a crab eating monkey was used to synthesize [^{3}H]17,20αP4 (X9) from [1,2,6,7-^{3}H]17OHP4 in the presence of 100 µM SU,and [^{3}H]16αOHP4 (X10) from [1,2,6,7-^{3}H]P4 in the absence of cofactors. All products were purified by HPLC.

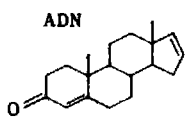
RESULTS (see also steroid scheme)

identification of X2 as 5,16-androstadien-3β-ol (ADL)



X2, a non-polar steroid formed from [^{14}C]P5 in human but not rat or monkey testicular homogenates, was identified as ADL as described in detail in chapter 3 using mass spectrometry as final evidence.

characterization of X1 as 4,16-androstadien-3-one (ADN)

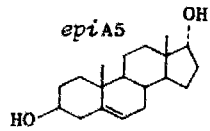


X1 is a highly non-polar metabolite formed from [^{14}C]P5 in human but not rat or monkey testicular homogenates. Evidence for X1 being identical to ADN is now based on several experimental data.It was the only metabolite formed from [^{3}H]ADL in human testicular homogenates. The synthesis of X1 from ADL could be inhibited by adding the 3βHSD inhibitors TRIL or DK to the incubation mixture whereas the cyt.P450 inhibitor SU had no effect. Furthermore, just like the synthesis of ADL from P5, the synthesis of X1 from P4 could be blocked by adding SU, but not by DK. X1 was not acetylatable.

characterization of X7 as 5-androstene-3β,17α-diol (epiA5)

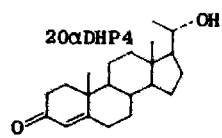
X7 is a polar metabolite appearing always together with ADL in human testicular homogenates (see chapter 5) and was characterized as *epi*A5. The synthesis of X7 from P5 could not be blocked by adding the

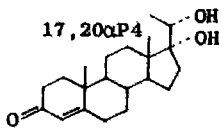
3βHSD inhibitor EPOS, suggesting it is a Δ5 steroid. After reduction of [1,2,6,7-^{3}H]DHEA with $NaBH_4$ 2 reaction products were formed (A5, 97% and *epi*A5, 3%) eluting with the same retention times as A5 and X7. Furthermore, the acetylation pattern indicated the presence of 2 acetylatable hydroxyl groups. When [^{3}H]X7 was used as substrate in a human testicular homogenate, a metabolite eluting with the same retention time as the Δ4 steroid *epi*T was formed, the synthesis of which could be blocked by the 3βHSD inhibitor EPOS.



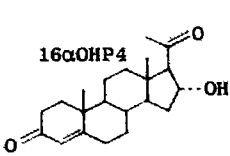
characterization of X4 and X9 as 20α-hydroxy-4-pregnen-3-one (20αDHP4) and 17α,20α-dihydroxy-4-pregnen-3-one (17,20αP4)

X4 and X9 are metabolites formed in high yields from [^{14}C]P5 in the monkey testicular homogenates. In the human testes they were formed under certain conditions as well (see chapter 4). X4 and X9 are probably identical to 20αDHP4 and 17,20αP4, the 20α-hydroxysteroid dehydrogenase metabolites of P4 and 17OHP4, respectively. Their chromatographic behaviour is in line with these proposals.Comparison of the profiles of products formed using various [^{3}H]steroids as substrate in the testicular homogenate of a crab eating monkey indicated P4 to be the direct precursor for X4 and 17OHP4 for X9. The presence of NADPH in the incubation medium, a prerequisite for 20αHSD activity [10], was essential for the synthesis of X4 from P4 and of X9 from 17OHP4. The cyt.P450 inhibitor SU (100 μM) did not interfere with these conversions whereas the 20αHSD inhibitor EPOS (100 μM) inhibited both. The acetylation patterns indicated the presence of 1 readily acetylatable hydroxyl group in both X4 and X9 (see experimental section).





characterization of X10 as 16α-hydroxy-4-pregnen-3,20-dione (16αOHP4)

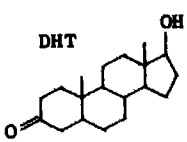


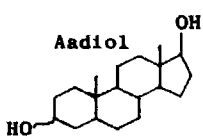
X10 is a highly polar metabolite formed from [^{14}C]P5 and [^{3}H]P4 in the monkey testis homogenates and from [^{3}H]P4 in the human testis homogenates. It eluted with the same retention time as 16αOHP4. The

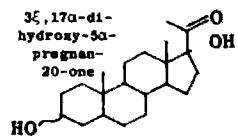
immediate precursor of X10 was P4. The synthesis of X10 from P4 was strongly inhibited by the cyt.P450 inhibitor SU (100 µM) but not by EPOS (100 µM). The acetylation pattern indicated the presence of 1 acetylatable hydroxyl group.

characterization of X3, X6, X8 and some other metabolites as 5α-reduced steroids

X3, X6 and X8 are metabolites formed from [^{14}C]P5 only in the testicular homogenates of rats. In Leydig cell cultures using [^{14}C]P5 (chapter 8) some other 'unidentified metabolites' were formed as well. All these metabolites reflect the well-known presence of 5α-reductase







activity in rat testes. X3, X6 and X8 eluted with the same retention times as DHT, Aadiol and 3ξ,17α-dihydroxy-5α-pregnan-20-one, respectively. In cultured rat Leydig cells some other 5α-reduced steroids were also found (chapter 8).

characterization of X5

X5 was synthesized in relatively low yields (usually 1-5%) in the testicular homogenates from humans, monkeys and rats. Its structure remains so far unknown.

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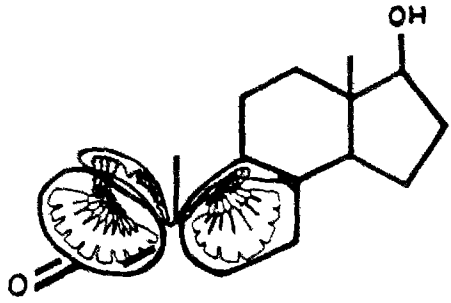
studies on some enzymatic conversions in testicular homogenates



part IIb

THE MECHANISM OF THE SYNTHESIS OF 16-ANDROSTENES IN HUMAN TESTICULAR HOMOGENATES.

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ABSTRACT

The biochemical pathway leading to the 16-unsaturated C_{19} steroids - known sex pheromone (precursors) in pig and man - is still a matter of dispute. In the 16-ene-synthetase process, via which 5,16-androstadien-3β-ol (ADL) or 4,16-androstadien-3-one (ADN) are biosynthesized from pregnenolone (P5) or progesterone (P4), a number of 2 or even 3 step conversions have been suggested in porcine testes, including 20β-reduction, 21-hydroxylation and 16,17-dehydrogenation.

Studying the 16-ene-synthetase reaction in *human* testicular homogenates, we adduced evidence for the hypothesis that ADL is synthesized from P5 in a single step, not requiring separate intermediates. Our proposal for the 16-ene-synthetase mechanism also explains why, at least in our hands, synthesis of ADL is always accompanied by co-synthesis of its satellite 5-androstene-3β,17α-diol (*epi*A5): both steroids are synthesized as a mere consequence of the fact that the proposed elimination and substitution reactions for the synthesis of ADL and *epi*A5, respectively, are competitive processes.

J Steroid Biochem, may 1989 (in press)

INTRODUCTION

The mechanism via which the 16-unsaturated C_{19} steroids - known sex pheromone (precursors) in pig and man [1,2] - are biosynthesized has been subject of discussion ever since their discovery in 1944 [3]. In early reports testosterone was found to be a precursor for these steroids [1,2,4-7]. Later, however, testosterone and a large number of other steroids including epitestosterone, testosterone-acetate, dehydroepiandrosterone, 16α-hydroxy-P4, 16α-hydroxy-P5, 17-hydroxy-P4, 17-hydroxy-P5, and 16-dehydro-P4 [1,2,8-13] have been excluded as precursors for 16-androstenes whereas P5 and P4 were found to be putative precursors [1,2,8,14-19]. In attempts to explain the 16-ene-synthetase process a number of conversions, including 20β-reduction (P5 → pregnenediol → ADL) [20], 21-hydroxylation (P4 → 21-hydroxy-P4 → ADN or P5 → 21-hydroxy-P5 → ADL) [10,21] and 16,17-dehydrogenation (P5 → 17-hydroxy-P5 → 16-dehydro-P5 → ADL) [13,22,23] have been suggested to occur prior to side chain cleavage in porcine testicular homogenates. A concerted process (P5 → ADL) has been suggested as well [11,19,24]. Some of the contradictory literature data concerning the possible intermediates (especially 17-hydroxy-P5) might be attributed to the different species used (immature or mature pigs or humans).

Detailed mechanistic studies on the 16-ene-synthetase reaction are scarce. Shimizu and Nakada [15,25,26] reported that 17-deuterated P5 was converted into ADL and into *epi*A5 by *boar* testicular microsomes with retention of the deuterium in both compounds. The deuterium was lost in the synthesis of androstenediol as was to be expected since this steroid was synthesized from P5 via 17-hydroxy-P5 and dehydroepiandrosterone, two intermediate steroids without hydrogen atoms at C-17. In a preliminary paper Osawa and Shibata [27] reported the retention of both 16β- and 17α-hydrogens and loss of the 16α-hydrogen in the conversion of P4 to 5α-androst-16-en-3β-ol.

In the present paper the 16-ene-synthetase reaction was studied using *human* testicular homogenates [19]. Comparison of our own data with those cited above led to a proposal for the reaction mechanism underlying the 16-ene-synthetase process. This proposal provides a satisfactory explanation as well for the experimental fact that, at least in our hands, the synthesis of ADL from P5 was always accompanied by the synthesis of *epi*A5.

MATERIALS AND METHODS

chemicals

[^{3}H]Steroids were purchased from New England Nuclear Corp. (Boston, MA) (P4, 57.0 Ci/mmol; 17-hydroxy-P4, 50.0 Ci/mmol; androstenedione, 85.0 Ci/mmol; testosterone, 41.6 Ci/mmol; androstenediol, 55.0 Ci/mmol) or from Amersham International (Amersham, UK) (P5, 19 Ci/mmol; 17-hydroxy-P5, 12 Ci/mmol; dehydroepiandrosterone, 60 Ci/mmol; 21-hydroxy-P4, 36 Ci/mmol). [4-^{14}C]P5 was purchased from Amersham International (56 mCi/mmol). Steroids were acetylated by dissolving them in a mixture of pyridine and acetic acid anhydride (25 µl each) and the reaction was stopped by adding 0.5 ml ethanol. [^{14}C]Pregnenediol was synthesized by reduction of [^{14}C]P5 with $NaBH_4$. [^{3}H]ADL and [^{3}H]*epi*A5 were synthesized by incubation of a human testicular homogenate with [^{3}H]P5 in the presence of the 3ß-hydroxysteroid dehydrogenase inhibitor cyanoketone [19]. The products were purified via HPLC (*vide infra*). Other chemicals were purchased from several commercial suppliers.

incubation, analysis and characterization of the metabolites

The techniques of homogenization, incubation, analysis and characterization of the metabolites have been described in detail elsewhere [19,28,29]. In short, testis tissue obtained from 5 patients who underwent orchidectomy for their prostatic carcinoma and from 2 patients with hydrocele and 3 with spermatocele who were biopsied during surgery, was homogenized (100 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose; 0°C) and centrifuged (10,000xg, 20 min, 4°C). The supernatant was incubated in a final volume of 1 ml with the radiolabeled steroid indicated in the presence of a NADPH-generating system and NAD (final concentrations, 0.4 mM NAD, 0.4 mM NADP, 4 mM glucose-6-phosphate, 0.12 U/ml glucose-6-phosphate dehydrogenase) in air in a shaking water bath at 32°C. The reaction was terminated by adding ice-cold diethylether. In the cases that ^{14}C-labeled substrate was used, ^{3}H marker steroids (P4, androstenedione, P5, dehydroepiandrosterone, testosterone, 17-hydroxy-P4, androstenediol, 17-hydroxy-P5, and estradiol) were added to monitor procedural losses. The

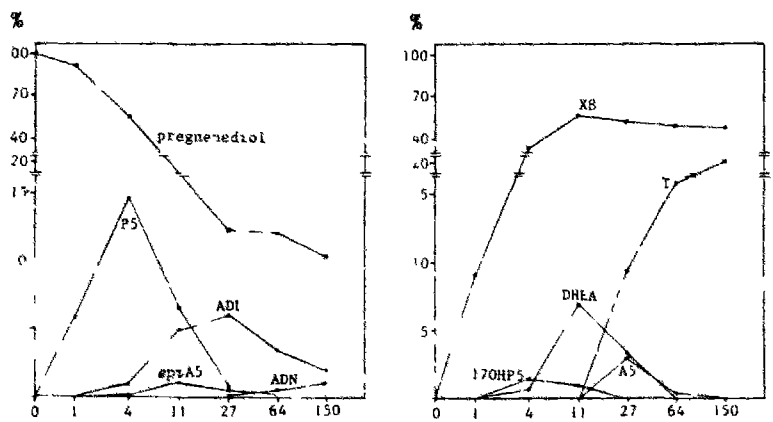
mixture was analyzed via HPLC using a silica aliphatic diol-column (Merck, Hibar LiChrosorb Diol 5 µm, 25x0.4 cm) with a n-hexane/ isopropanol gradient [28] and fractionation in 154 scintillation vials (3/min), enabling separation of a large number of steroids in a single run [19,29]. The recoveries of the [^{3}H]marker steroids were usually 45-65%. In the cases that ^{14}C labeled substrate was used, yields are expressed as percentage of total amount of radioactivity added as [^{14}C]substrate. In the cases that ^{3}H labeled substrate was used yields are expressed as percentage of total recovered radioactivity.

In an earlier paper [28] we reported the synthesis of several 'unidentified metabolites' from [^{14}C]P5. For this paper only the metabolites designated X1, X2 and X7, that were formed in human but not in rat [28] or monkey [29] testes, are of interest. The identification of X2 as ADL, using mass-spectrometry as final evidence, had been described in detail elsewhere [19]. Evidence for X1 being identical to ADN is now based on several experimental data. The conversion of [^{3}H]ADL to X1 by human testicular homogenates could be inhibited by adding the 3β-hydroxysteroid dehydrogenase inhibitor cyanoketone. Like the conversion of P5 to ADL [19], the conversion of [^{3}H]P4 to X1 could be blocked by adding the cytochrome P450-inhibitor SU-10603, but not by cyanoketone. X1 was not acetylatable. Evidence for X7 being identical to *epi*A5 [19] is also based on several experimental data. The pattern of products formed after acetylation of [^{3}H]X7 using different reaction times indicated the presence of 2 different, acetylatable hydroxyl groups. Furthermore, after reduction of [^{3}H]dehydroepiandrosterone with $NaBH_4$ both the 17-epimeric androstenediols were formed, eluting in our HPLC system with the same retention times as androstenediol and X7. When [^{3}H]X7 was used as substrate in a human testicular homogenate, a metabolite eluting with the same retention time as epitestosterone, the 3β-hydroxysteroid dehydrogenase metabolite of *epi*A5, was formed, the synthesis of which could be blocked with cyanoketone.This 3β-hydroxysteroid dehydrogenase inhibitor did not interfere with the conversion of P5 to X7, indicating X7 bears the 3β-hydroxy-5-ene moiety.

RESULTS

Human testicular homogenates were incubated with several radiolabeled steroids to study their ability to serve as precursor for ADL or ADN. [^{14}C]P5 and [^{3}H]P4 were converted to 16-androstenes in yields of 20-25% [19] and about 2%, respectively, whereas the [^{3}H]C_{19} steroids dehydroepiandrosterone, androstenediol, androstenedione, and testosterone and the [^{3}H]C_{21} steroids 17-hydroxy-P5 and 17-hydroxy-P4 were not. The [^{3}H]steroids *epi*A5 and 21-hydroxy-P4 were not converted into ADL or ADN either. These data indicate that hydroxylations at the 17α- or 21-positions are not involved in the 16-ene-synthetase process. [^{14}C]Pregnenediol was converted to ADL and ADN, but the time sequence study suggested it was first oxidized to P5 and subsequently to ADL (fig 1). Detectable amounts of *epi*A5 were synthesized as well. Remarkably, huge amounts of a very polar metabolite designated Xß were formed within 1 min. The nature of this metabolite remains unknown but the finding that Xß was not formed in detectable amounts using [^{14}C]P5 as substrate provides additional evidence for the idea that in the

Fig 1. Time sequence in the metabolism of [^{14}C]pregnenediol. Left panel: substrate and metabolites of the 16-ene-synthetase process. Right panel: other metabolites. Yields are expressed as percentage of total amount of radioactivity added as [^{14}C]substrate.



A5, androstenediol; DHEA, dehydroepiandrosterone; T, testosterone; 17OHP5, 17-hydroxy-P5; Xß, unidentified metabolite.

human testis pregnenediol is not an intermediate in the conversion of P5 to ADL. Since Xß was not found using [^{14}C]P5 as substrate, no efforts were done to characterize this metabolite.

The possible effects of 16-dehydro-P5 on microsomal steroid metabolism were studied by incubating a human testicular homogenate with [^{14}C]P5 and 100 µM radioinert 16-dehydro-P5. After 150 min of incubation the only quantitatively important ^{14}C labeled metabolite formed was P4 (about 10%), whereas only minor amounts of 17-hydroxy-P4 and 17-hydroxy-P5 were detected as other metabolites (both about 1%). In the presence of 10 µM 16-dehydro-P5 the same effects, but to a lesser extent, were found. These data indicate that 16-dehydro-P5 interfered with steroid metabolism, especially with cytochrome-P450 linked reactions.

In all experiments performed on human testicular homogenates in our laboratory, ADL and *epi*A5 were formed in combination with each other; all efforts to suppress the synthesis of one without affecting the other using various chemicals (SU-10603, cyanoketone, epostane, cyproterone, aminoglutethimide, metyrapone, spironolactone, tamoxifen, hydroxycholesterols, 16-dehydro-P5, ADL, 17-hydroxy-P5 and others) failed [19,29, and unpublished data]. It has to be noted that in none of these experiments evidence was found for the synthesis of 21-hydroxy-P5, pregnenediol, or any other metabolite that might be an intermediate in the 16-ene-synthetase reaction. The data presented in this paper and those of others (see Introduction) indicate that the biosynthetical interrelations between P5, P4, ADL, ADN and *epi*A5 are as presented in fig 2.

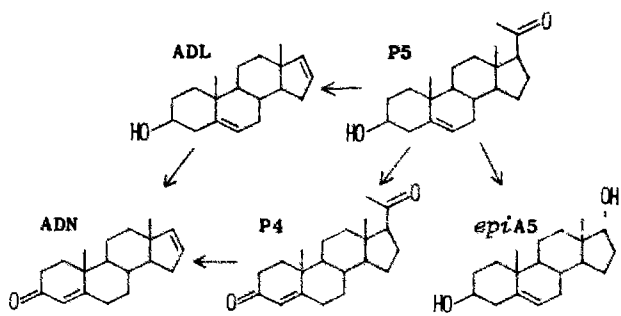
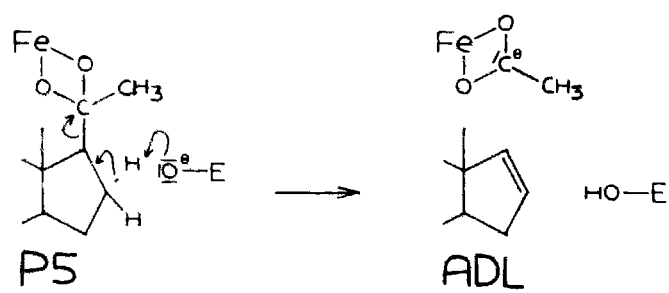


Fig 2. Pathways in the 16-ene-synthetase process.

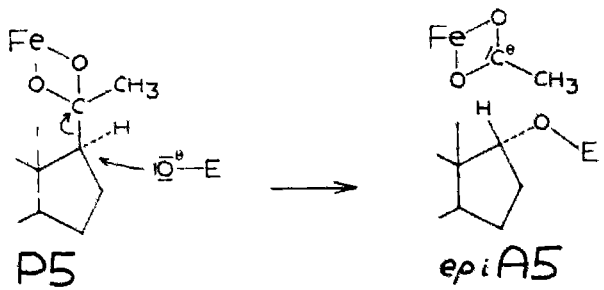
DISCUSSION

In this paper the 16-ene-synthetase reaction has been studied in detail using human testicular homogenates. In our hands, ADL and *epi*A5 were always formed in combination with each other. In testicular homogenates of rats and macaques, using identical conditions, ADL, ADN and *epi*A5 were not found, regardless the substrate used [19,28,29]. Furthermore, during the first 30 min of incubation of human testicular homogenates with [^{14}C]P5, in which the metabolism of ADL and *epi*A5 remained negligible, the ratio ADL/*epi*A5 was roughly constant in every experiment [19]. Shimizu [26] reported the same phenomenon for boar testes. In addition, we found no evidence for the existence of intermediates in the conversion of P5 to ADL. These data suggest that both ADL and *epi*A5 are synthesized from P5 by a single enzyme via competing mechanisms. Taking into account that the 17α-hydrogen is maintained in the conversion of P5 to ADL and *epi*A5 [15,25-27], the following proposal was formulated. P5 binds to the iron atom of cytochrome P450 via its carbonyl at C-20. The steroid nucleus is bound to the porphyrin nucleus via hydrophobic interactions while the hydroxyl at C-3 is bound via electrostatic interactions to the propionyl side chains of the porphyrin nucleus [30]. ADL is then synthesized via an elimination reaction that is initiated by a nucleophilic attack of the enzyme at the 16α-hydrogen of P5; abstraction of the 16β-hydrogen is not allowed since the elimination reaction can proceed only when the eliminated groups are in the *anti*-configuration:



The negatively charged 'O' in this drawing represents an oxygen atom of one of the amino acid residues in the active site of the enzyme 'E' or an hydroxylic anion that is stabilized in this site. The synthesis of *epi*A5 proceeds via a competing mechanism, i.e. via a nucleophilic

substitution that is initiated by a nucleophilic attack at C-17:



In both cases the hydrogen atom on C-17 is maintained whereas the 16α-hydrogen is abstracted in the synthesis of ADL (see Introduction).

This proposal for the 16-ene-synthetase mechanism is an illustration of the principle that although enzymes are specific biochemical catalysts, they are subjected to all rules of organic chemistry. The substrate specificity of the reaction appeared to be high, as reflected by the large difference in yields of 16-androstenes using P5 or P4 as substrate (20-25% and about 2%, respectively). Nevertheless, synthesis of *epi*A5 from P5 can not be prevented since the elimination and substitution reactions are competitive processes. The proposal indicates that *epi*A5 is a side-product of the 16-ene-synthetase reaction and not a 'pre-selected end-product'. A physiological role of this steroid (and its better documented metabolite epitestosterone [31,32]) is not known but might, in this way, not exist at all. Another remarkable consequence of the proposal is that *epi*A5 is synthesized from P5 in a single step whereas synthesis of androstenediol is known to proceed via 2 different intermediates (17-hydroxy-P5 and dehydroepiandrosterone).

It is important to stress that the fact that ADL and *epi*A5 appeared only in combination with each other can not be explained by non-specific destruction of one steroid yielding the other since in incubations of testicular homogenates with [^{3}H]ADL no *epi*A5 was formed and *vice versa* [29].

The suggestion of Loke and Gower [20] that pregnenediol might be an intermediate in the 16-ene-synthetase reaction is rather puzzling as the conversion of P5 to pregnenediol is a reductive process whereas the conversion of P5 to ADL is oxidative. The time sequence studies, however, indicated that pregnenediol was first converted to P5 and subsequently to ADL and *epi*A5 (fig 2). The synthesis of T proceeded

via the same pathway as found previously using [^{14}C]P5 as substrate [28,29], indicating that the microsomal conversions were not influenced by the relatively high concentrations of P5 in these experiments. Our proposal excludes pregnenediol as immediate precursor of ADL since the C-20 carbonyl plays an important role during the reaction in binding P5 to the cytochrome P450 nucleus.

The suggestion of Mason *et al* [22] that 16-dehydro-P5 might be an intermediate in the conversion of P5 to ADL in neonatal pig testicular microsomes was based on few experimental data. The authors used a single TLC-separation and identified one of their metabolites formed from [^{14}C]P5 as 16-dehydro-P5 by co-chromatography and recrystallization to constant specific activity. These data alone, however, are not sufficient to characterize a steroid completely [33]. Kwan *et al* [23] were not able to confirm synthesis of 16-dehydro-P5 from P5 using similar conditions. Furthermore, the sequence proposed by Mason *et al* [22] (P5 → 17-hydroxy-P5 → 16-dehydro-P5 → ADL) was entirely based on the temporal sequence of occurrence of the metabolites; an experiment using radiolabeled 17-hydroxy-P5 as substrate was not performed. The possibility that ADL might be synthesized directly from P5 at much lower reaction rates than 17-hydroxy-P5 can not be excluded and might even be of physiological importance since it seems natural that pheromone production has to remain limited before maturation. Furthermore, the bond between carbons 17 and 20 is more stable in 16-dehydro-P5 than in P5 due to the conjugation of the 16-17 double bond with the carbonyl at C-20 in 16-dehydro-P5. As a result synthesis of ADL will proceed, from a thermodynamic point of view, easier from P5 than from 16-dehydro-P5.

When radioinert 16-dehydro-P5 was added to a human testicular homogenate the metabolism of [^{14}C]P5 was strongly inhibited, especially the cytochrome-P450 linked reactions. This inhibition might be explained by the fact that due to the 20-keto-16-ene moiety of 16-dehydro-P5 this steroid can bind with a higher affinity to the cytochrome-P450 iron atom than P5. This finding might be important in the interpretation of the results of the experiments by Kwan *et al* [13,34] who incubated neonatal pig testicular microsomes with 250 µM radioinert 16-dehydro-P5 and concluded that this steroid was an intermediate in the synthesis of ADL.

Interestingly, essentially the same mechanism as proposed in this

paper for the conversion of P5 to ADL can be used to describe one step in the biosynthesis of cholesterol, i.e. the 14α-demethylation of lanosterol [35,36], and the C17,20-lyase reaction. In the first process the 15ß-hydrogen of the 32-oxo-metabolite of lanosterol is attacked. In the latter process, converting 17-hydroxy-P5 to dehydroepiandrosterone or 17-hydroxy-P4 to androstenedione, the atom to be subjected most likely to the nucleophilic attack is the hydrogen of the 17α-hydroxylgroup. In both reactions the hydroxylated derivates will not be formed (primarily due to sterical hindrance).

In conclusion, the data presented in this paper support our hypothesis that ADL is synthesized from P5 in human testicular homogenates in a single step without separate, isolatable intermediates. The proposal for the 16-ene-synthetase mechanism also explains the synthesis of *epi*A5; this steroid is synthesized because elimination and substitution reactions are competitive processes. The 17α-hydrogen of P5 is maintained in the synthesis of both ADL and *epi*A5 whereas the 16α-hydrogen is lost in the synthesis of ADL.

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LIST OF ABBREVIATIONS

ADL, 5,16-androstadien-3ß-ol
ADN, 4,16-androstadien-3-one
androstenedione, 4-androstene-3,17-dione
androstenediol, 5-androstene-3ß,17ß-diol
cyanoketone, 2α-cyano-17ß-hydroxy-4,4,17α-trimethyl-5-androsten-3-one
16-dehydro-P4, 4,16-pregnadiene-3,20-dione
16-dehydro-P5, 3ß-hydroxy-5,16-pregnadien-20-one
dehydroepiandrosterone, 3ß-hydroxy-5-androsten-17-one
*epi*A5, epiandrostenediol, 5-androstene-3ß,17α-diol
epitestosterone, 17α-hydroxy-4-androsten-3-one
P4, progesterone, 4-pregnene-3,20-dione
P5, pregnenolone, 3ß-hydroxy-5-pregnen-20-one
pregnenediol, 5-pregnene-3ß,20ß-diol
SU-10603, 7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphtalenone
testosterone, 17ß-hydroxy-4-androsten-3-one

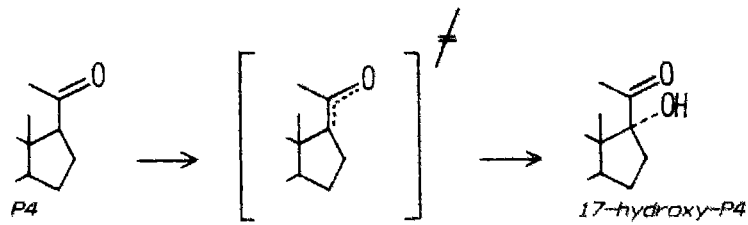
ADDITIONAL DATA ON CYTOCHROME P450-LINKED ENZYMES

As already alluded to in chapter 5, the proposal for the 16-ene-synthetase reaction can be applied without modifications to the lyase reaction as well, leaving room for the possibility that the enzyme might be capable of catalyzing both reactions. Although it is now generally accepted that 17αOHase and lyase activities are associated with the same protein [1-12], little is known about the ability of this protein to exert 16-ene-synthetase activity as well. Numerous authors purified and studied porcine testicular 17αOHase/lyase but did not report that their preparation exerted 16-ene-synthetase activity as well [1,13-15], or that a 16-ene-synthetase preparation contained 17αOHase or lyase activity [16,17]. However, Nakajin *et al* [18] showed that their 17αOHase/lyase preparation did contain 16-ene-synthetase activity, but only in the presence of cyt.b5. They therefore concluded that 17αOHase, lyase and 16-ene-synthetase activities are probably all associated with the same protein. The porcine 17αOHase/lyase preparations mentioned above might have been devoid of 16-ene-synthetase activity due to the absence of cyt.b5, but the isolation of 16-ene-synthetase without 17αOHase or lyase activity is less easy to explain if all reactions are indeed catalyzed by the same enzyme. The finding that a single antiserum was able to inhibit both 17αOHase/lyase and 16-ene-synthetase activities in neonatal porcine testicular microsomes [18] is in itself not very surprising since many cyt.P450 enzymes are structurally closely related [19,20]. The fact that a single purified enzyme can catalyze different reactions *in vitro* does not necessarily imply that it exerts all activities *in vivo* as well, since the ultimate tertiary structure, which is dictated by the primary structure of the enzyme but also by its surroundings and/or the presence of other compounds (such as cyt.b5), determines the biological activity [18, 21-24]. In this respect it is interesting to note that 17αOHase/lyase obtained from pig adrenal microsomes [2,3,22] was capable of catalyzing both reactions *in vitro* whereas *in vivo* the lyase activity must be suppressed since cortisol is the major steroid secreted by this gland. Also, in patients with lyase deficiency [25,26] extremely high circulating levels of 17OHP5 and 17OHP4 were found, indicating that 17αOHase activity was not deficient. Furthermore, our experiments on

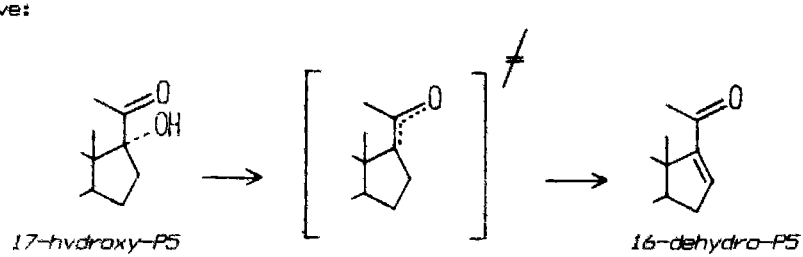
the effects of several compounds on [^{14}C]P5 metabolism in testicular homogenates indicate that 17αOHase and lyase and also 16-ene-synthetase activities do have different properties *in vitro*. For example, the alleged 3βHSD inhibitor EPOS (chapter 8) at 100 μM concentration inhibited the lyase reaction as 17OHP5 accumulated over DHEA and A5 whereas the 16-ene-synthetase and 17αOHase reactions remained virtually unaffected. SU (chapter 8), at 10 μM, prevented the synthesis of ADL and other C_{19} steroids, but after 150 min incubation 17OHP4 was clearly measurable, indicating the 17αOHase reaction was not inhibited completely. Similar differential effects were noticable for metyrapone, spironolactone, tamoxifen, clomiphene and 16-dehydro-P5 (data not shown). Only the synthesis of ADL and *epi*A5 (X7) remained coupled under all conditions. Finally, it has to be noted that in our experiments on cultured human Leydig cells (chapter 7) it was found that all biosynthetic precursors of T were excreted in the incubation medium; even P5 and 17OHP5 reached considerable levels. Apparently, the conversion of P5 to DHEA proceeded in two separate steps: P5 was converted to 17OHP5 by a 17α-hydroxylating 17αOHase/lyase enzyme, and subsequently converted to DHEA by another 17αOHase/lyase enzyme, catalyzing the lyase reaction. If one single 17αOHase/lyase enzyme would catalyze both reactions, 17OHP5 would remain bound to the enzyme and would not be released in the incubation medium.

In chapter 5 a proposal for the mechanism of the 16-ene-synthetase reaction has been presented and some reference was made to the possible mechanism of the lyase reaction. Data suggesting a possible mechanism of the 17αOHase reaction were obtained in experiments using the testicular homogenate of a crab eating monkey. In these experiments [^{14}C]20αDHP4 (X4) was used as substrate and was found to remain unmetabolized during 150 min of incubation; its 17α-hydroxylated derivate 17,20αP4 was not found. These data are in line with our finding that levels of 20αDHP4 (X4) that were formed from [^{14}C]P5 in the monkey testes reached plateaus when the supply of P4 was exhausted (chapter 4 fig 3) and indicate that the 20α-hydroxylated steroid did not serve as substrate for 17αOHase. Apparently, the C-20 carbonyl of P4 is essential in the 17αOHase reaction. It has to be admitted that this conclusion is not entirely surprising, since in the conversion of P4 to 17OHP4 the 17α-hydrogen of P4 has to be replaced by a hydroxyl-group with retention of the stereochemical configuration. For this

reaction to occur the hydrogen has to be removed first since carbon lacks *d*-orbitals in its valence shell. The remaining charge (or electron in case of intermediate radicals) will be stabilized by the adjacent C-20 carbonyl:



Obviously, exactly the same principle will be valid for the conversion of P5 to 17OHP5, but it can be applied to a step in the sequence proposed by Mason *et al* [27] for the conversion of P5 to ADL in neonatal porcine testicular microsomes as well. The authors suggested a pathway via 17OHP5 and 16-dehydro-P5 (discussed in chapter 5). In the conversion of 17OHP5 to 16-dehydro-P5 it seems obvious that the 17α-hydroxyl is removed first, yielding the same transition state as mentioned above:



Since both steps (P5 → 17OHP5 and 17OHP5 → 16-dehydro-P5) are, in this way, likely to proceed via identical transition states, 17OHP5 is not an obligatory intermediate in the conversion of P5 to 16-dehydro-P5 and the pathway can be depicted simply as P5 → 16-dehydro-P5.

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chapter 6

THE MECHANISM OF THE Δ5-3-KETOSTEROID ISOMERASE REACTION

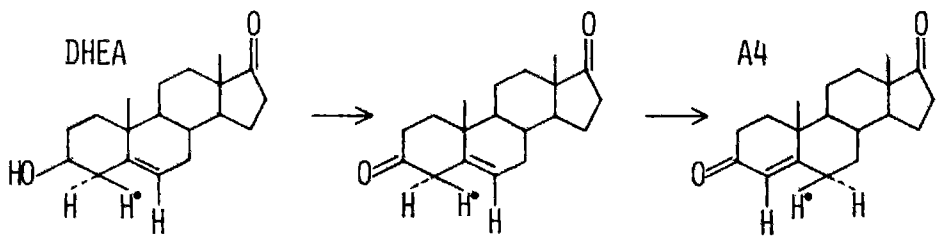
ABSTRACT

The mechanism of the Δ5-3-ketosteroid isomerase reaction, the 2nd step in the conversion of Δ5-3β-hydroxysteroids to Δ4-3-ketosteroids via the corresponding Δ5-3-ketosteroids, was investigated. The data indicate that the reaction in testicular homogenates of rat, monkey and man is likely to proceed via an intramolecular shift of the 4β-hydrogen of the Δ5-3-ketosteroid to the 6β-position of the Δ4-3-ketosteroid.

INTRODUCTION

The Δ5 steroids are converted to the corresponding Δ4 steroids via a 2-step reaction [1-4]. For example, the Δ5-3ß-hydroxysteroid 3ß-hydroxy-5-androsten-17-one (DHEA) is converted first into the Δ5-3-ketosteroid 5-androstene-3,17-dione by Δ5-3ß-hydroxysteroid dehydrogenase. In spite of the fact that the latter metabolite will isomerize to the Δ4-3-ketosteroid 4-androstene-3,17-dione (A4) spontaneously in aqueous solutions, Δ5-3-ketosteroid isomerase activity is present in steroidogenic tissues to catalyze this reaction. The existence of this

Fig 1. The conversion of DHEA to A4 via 5-androstene-3,17-dione



isomerizing enzyme has first been reported in 1955 for *Pseudomonas testosteroni* [5]. In this species the dehydrogenase and isomerase activities were separable [1,5], but in mammalian steroidogenic tissues (especially adrenals and testes) both activities were found to be associated with a single protein [2-4,6-9]. The bovine adrenal appeared to be an exception [10-12]. For *P. testosteroni* [1] the isomerase reaction has been investigated in great detail and the authors showed that the isomerization of 5-androstene-3,17-dione to 4-androstene-3,17-dione (A4) proceeds via an intramolecular shift of the 4ß-hydrogen to the 6ß-position. In this chapter we investigated whether the isomerization of Δ5-3-ketosteroids to Δ4-3-ketosteroids in rat, monkey and human testicular homogenates proceeds via an intramolecular hydrogen-shift as well.

EXPERIMENTAL

The radiolabeled steroid [4,7-^{3}H]P5 bears a tritium on C-4. One of the hydrogens of C-4 will be lost when the conversion to P4 does not

proceed via an intramolecular hydrogen shift. To be able to determine this possible ^{3}H-loss, testicular homogenates of a rat, crab eating monkey and prostatic carcinoma patient were incubated with a mixture of [4,7-^{3}H]P5 and [4-^{14}C]P5 (for experimental details see chapter 2). If ^{3}H is lost the $^{3}H/^{14}C$ ratio of P5 will be higher than that of P4, if not, the ratios will be the same. The incubations were performed in the presence of SU (100 µM) to inhibit 16-ene-synthetase, 17αOHase and 16αOHase activities whereas no NADP(H) was added to prevent synthesis of 5α-reduced or 20α-hydroxylated derivates (especially 20αDHP4 (X4)). In this way the possible conversions are limited to P5 → P4. Incubations were performed using different reaction times and the $^{3}H/^{14}C$ ratios of the metabolites were determined using a 10-min counting time.

RESULTS AND CONCLUSION

The results of the experiments are presented in table 1 and indicate that no ^{3}H was lost in the conversion of P5 to P4 as virtually no differences were found in the $^{3}H/^{14}C$ ratios of P4 and P5 in the species tested. By the way of reasoning described above, the Δ5-3-keto-

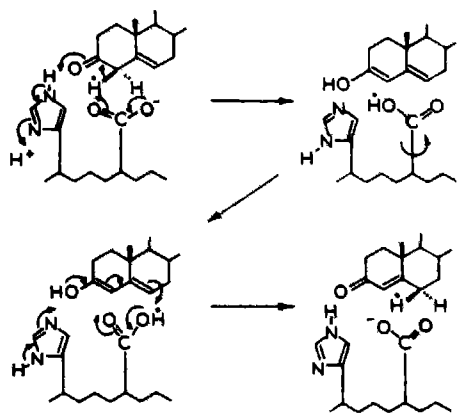
Table 1. $^{3}H/^{14}C$ ratios of P5 and P4 in testicular homogenates of different species after 26 or 150 min of incubation.

species	time	$^{3}H/^{14}C$	
		P5	P4
man	26	0.687	0.695
	150	0.680	0.689
monkey	26	0.661	0.674
	150	0.725?	0.673
rat	26	0.676	0.672
	150	0.668	0.670

steroid isomerase reaction is very likely to proceed in testicular homogenates of the rat, crab eating monkey and man via a similar mechanism as described for *Pseudomonas testosteroni*, i.e. via an intramolecular shift of the 4ß-hydrogen to the 6ß-position. For this bacterium Batzold *et al* [1] indicated that a histidine and aspartate

residu participated in the reaction as shown in fig 2. It remains to be determined whether the same amino acid residues are involved in the isomerase reaction in the testicular homogenates studied.

Fig 2. The mechanism proposed by Batzold et al *for the Δ5-3-keto-steroid isomerase reaction in* P. testosteroni. *Reproduced from reference [1].*



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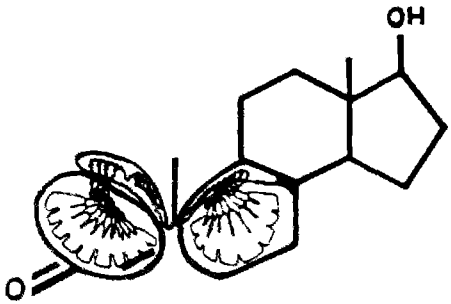
experiments on leydig cell culture



part III

HUMAN LEYDIG CELLS IN CULTURE. I. ISOLATION TECHNIQUE AND STEROIDOGENIC RESPONSE TO HUMAN CHORIONIC GONADOTROPIN

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ABSTRACT

A simple technique for the isolation and culture of rat and human Leydig cells was developed and the steroidogenic response to increasing doses of hCG was studied by measuring the concentrations of the 8 steroids of the Δ4 and Δ5 biosynthetic pathways leading from pregnenolone to testosterone. In both man and rat the Leydig cells responded in a dose dependent fashion to hCG but human Leydig cells were 10 to 100-fold less sensitive. In both species steroid production increased to 5 to 20 times the baseline levels. In rats high amounts of the Δ4 steroids androstenedione and testosterone were synthesized in response to hCG whereas in the human Leydig cell cultures the Δ5 steroid dehydroepiandrosterone was quantitatively the most important steroid formed. The Leydig cell cultures studies for the first time demonstrate that in the human testis the Δ5 route is indeed the main pathway of steroid biosynthesis, confirming data obtained in testicular homogenates.

J Androl. submitted

INTRODUCTION

Several studies reported that *in vivo* administration of hCG to eugonadal men evokes a biphasic response in peripheral testosterone (T) levels with a modest (20-60%) increase within 6 hr followed by a 2 to 3 fold rise at 48 to 72 hr [1-5]. In rats, however, increments 10 to 20 times the baseline value occur within 6 hr after hCG [1,2]. *In vitro* Huhtaniemi *et al* [6] confirmed the poor Leydig cell responsiveness to hCG in man as compared to the precipituous rise in rats. Recently, however, Simpson *et al* [7] using isolated human Leydig cells highly purified via density gradient centrifugation, demonstrated that the function of human Leydig cells may be more similar to that of rats than thought previously. Unfortunately, only the T response to hCG was studied. These controversial data prompted us the study the steroidogenic response to hCG in cultured human and rat Leydig cells obtained via a simple isolation technique without previous percoll purification, with special emphasis on the preferred pathways in the biosynthesis of T.

MATERIALS AND METHODS

testis tissue

Human testis tissues were obtained from 2 patients, aged 61 and 68 yr, who underwent subcapsular orchiectomy for their prostatic carcinoma. None of them recieved medication known to interfere with steroidogenesis. Rat testes were obtained from 2 adult Wistar rats, weighing about 200 gr, killed by cervical dislocation. Testis tissue was transferred to 0.9% NaCl and used immediately.

isolation and culture of Leydig cells

Leydig cells were isolated essentially according to the methods described elsewhere [7-9]. The culture medium consisted of MEM (Gibco, Breda, The Netherlands; Cat. No. 041-01095) supplied with 1% non-essential amino acids (Gibco, 043-01140), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, 061-05145) and 0.6 µg/ml fungizone (Gibco, 061-05295). Rat testes were decapsulated carefully without cutting or

teasing the tissue, while human testis tissue was cut into little pieces. About 3 gram testis tissue was transferred to 50-ml plastic tubes (Greiner, Alphen aan de Rijn, the Netherlands) and 7 ml culture medium containing 200 U/ml collagenase (Sigma, St. Louis, MO; type I, 260 U/mg) were added. The tubes were shaken longitudinally (80 cycles/min) in a shaking water bath of 32°C for 30 (rat) or 45 (human) min. About 20 ml 0.9% NaCl were added and the tubes were inverted several times. Non-dispersed tissue was allowed to settle while the tubes remained vertical for about 3 min. The supernatant was filtrated through one layer of nylon gauze (Monodur, Stokvis en Smit's Textielmaatschappij, Haarlem, The Netherlands; 60 μm) and the filtrate was centrifuged at room temperature for 10 min at 150x*g*. The cells were washed twice with culture medium and were finally resuspended in culture medium containing 1% FCS (Gibco, batch 100265 1A) (about 2.10^6 nucleated cells per ml).

Five hundred μl of the cell suspension (about 10^6 nucleated cells) were pipetted onto culture dishes (Lux, 35x10 mm, Flow Laboratories, Amstelstad, Amsterdam, The Netherlands) containing 500 μl culture medium with 1% FCS. The cells were subsequently preincubated for 1 (rat) or 2 (human) hr in a humidified atmosphere (5% CO_2, 95% air) at 32°C. The medium and floating cells were removed by suction and the cells attached to the dishes were washed twice with 1 ml serum-free culture medium. Then 1 ml culture medium containing hCG (Pregnyl, Organon, Oss, The Netherlands) in the concentrations indicated (see fig 1) was pipetted onto the dishes and the cells were incubated in a humidified atmosphere (5% CO_2, 95% air) at 32°C. The medium was then pipetted into 5-ml borosilicate glass tubes and stored at -20°C prior to analysis.

RIA procedure

The concentrations of steroids in the culture media were determined via RIA procedures after chromatographic purification of the steroids. ^{3}H-Labeled P5, 17OHP5, DHEA, A5, P4, 17OHP4, A4, and T (about 10^4 dpm each) were added to the samples to monitor procedural losses. They were extracted with diethyl ether and the 8 steroids mentioned were separated completely from each other via HPLC in a single run, using a silica aliphatic diol column (Merck Hibar LiChrosorb Diol 5 μm, 25x0.4

cm) with a n-hexane/isopropanol gradient [10,11]. After purification an aliquot was used to determine the recovery. The samples and standard curves in duplo (0 - 5000 fmol/tube) were incubated overnight at 4°C in the presence of antiserum and [^{3}H]tracer and the bound and free fractions were subsequently separated by dextran coated charcoal [12-14]. An aliquot of the antibody-bound fraction was assayed for ^{3}H in a liquid scintillation counter. The steroid concentrations in the samples were calculated using the 4-parameter linearization method of Healy [15,16] for the standard curve. The concentrations were corrected for the extra amount of steroid added as recovery tracer.

RESULTS

The concentrations of the 8 different steroids of the Δ4 and Δ5 pathways (P4, 17OHP4, A4, T, P5, 17OHP5, DHEA, and A5) in the culture media that were synthesized in response to increasing concentrations of hCG by cultured human and rat Leydig cells are presented in fig 1. As can be derived from this figure, qualitatively and quantitatively large differences were found between the steroidogenic responses of human and rat Leydig cells, but quantitatively also between the two rats studied. The rat cells responded to much lower levels of hCG than the human, indicating a 10 - 100 fold reduction in sensitivity of the human Leydig cells as compared to the rat. Both species were found to be almost non-responsive below an apparently critical concentration. At higher concentrations of hCG the quantitatively most important steroid formed in the rat cell cultures was T, but in the human DHEA. In the rat Leydig cells the Δ5 steroids DHEA and A5 did not reach detectable levels. High levels of the Δ4 steroid A4 were formed by the rat cells, rising in concert with T, whereas in the human cells only a minor rise of A4 was found. In the rat cells the Δ4 steroids were quantitatively the most important, with 17OHP5 as only Δ5 intermediate, whereas in the human cells high amounts of Δ5 steroids were found with T and 17OHP4 as only important Δ4 steroids. Interestingly, in the human Leydig cells T and A5 reached plateaus at hCG concentrations exceeding 0.3 IU/ml, in contrast to DHEA and its precursors, indicating that the 17-ketosteroid oxidoreductase catalyzed conversion of DHEA to A5 was rate limiting.

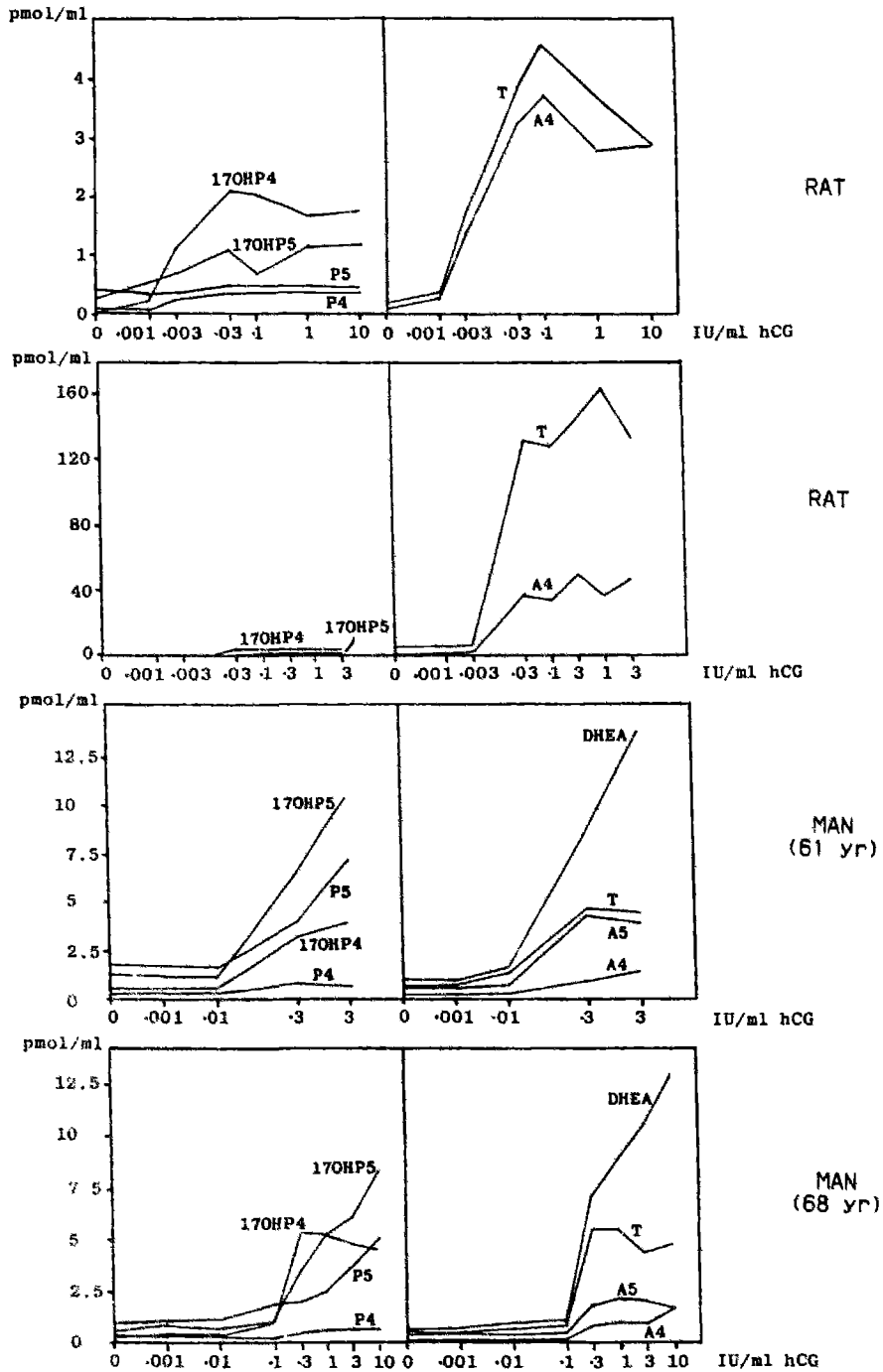

Fig 1. Steroidogenic response to hCG of cultured rat and human Leydig cells. Each point in the figure represents the mean of duplicate determinations. Standard deviations (<15% of the concentrations) have been omitted for clarity.

DISCUSSION

In this paper the steroidogenic response to hCG of cultured human and rat Leydig cells is described. The data clearly indicate that isolation and culture of human Leydig cells is possible via a simple isolation technique, without purification via density gradient centrifugation as is frequently used for rat Leydig cells [7-9,17-19]. We presented the data on 2 patients, in whom the concentrations of all major Δ5 and Δ4 steroid were determined. In these two experiments, but also in 6 others in which only T production was measured via a direct RIA (data not shown), the cells were highly responsive and hCG-stimulated steroid production 5 to 20 times above baseline was found regularly. For rat Leydig cells these results could be expected, since numerous authors found similar responses to LH or hCG [7,8,17-19]. In contrast, for human Leydig cells several authors reported poor T responsiveness after *in vivo* administration of hCG [1-5], but also after *in vitro* stimulation of cultured Leydig cells (Huhtaniemi *et al* (1982) [6]). Very recently Simpson *et al* [7], however, also reported 5 to 17-fold stimulation of T production by cultured human Leydig cells, but only after extensive purification of the cells. The reason for the discrepancy between Huhtaniemi's and Simpson's and our results is unknown, but one of the important factors might very well be the temperature used during the collagenase dispersion: Huhtaniemi *et al* [6] dispersed at 35°C, Simpson *et al* [7] and we at 32°C. It is well-known that Leydig cells are heat sensitive [20-22]. Obviously, the low responsiveness found *in vivo* can not be explained in this way. Although the magnitude of T responsiveness to hCG in human cells was comparable to that of rats, the human Leydig cells were 10 to 100 fold less sensitive to hCG stimulation. These results are in agreement with those of Simpson *et al* [7]. These authors stated that the lower sensitivity may be due to the fact that testicular tissue was obtained from aging men who have raised plasma LH levels and thereby a decreased number of LH receptors.

The profiles presented in fig 1 indicate that large differences exist between human and rat Leydig cells, not only in sensitivity but mainly in the steroidogenic pathways leading to the synthesis of T. This conclusion is in contrast to that of Simpson *et al* [7], who concluded that human and rat Leydig cells are probably more similar

than thought previously, since in both species similar fractionation patterns on a discontinuous percoll gradient were found and similar responsiveness of T to hCG. We also found similar stimulation factors in human and rat Leydig cells, but our detailed data on the steroidogenic response to hCG for the first time reveal that in the rat Leydig cells high amounts of Δ4 steroids were formed, especially A4 and T, whereas in the human Leydig cells the Δ5 steroids were quantitatively much more important, especially DHEA. These data confirm our earlier published results concerning the preferred pathways leading from P5 to T obtained in human and rat testicular homogenates [10]. Interestingly, in the human Leydig cell cultures levels of A5 and T reached plateaus at the higher hCG concentrations, whereas DHEA and its precursors did not, indicating that 17-ketosteroid oxidoreductase activity may be rate limiting in the biosynthesis of T.

As can be derived from fig 1, determination of T production alone, via a direct RIA or after chromatographic purification, results in an underestimation of the total steroidogenic output of the cultured Leydig cells, especially the human. For human Leydig cell cultures a direct RIA for DHEA will yield more reliable information concerning the Leydig cell steroidogenic capacity, especially if the antiserum displays enough cross-reactivity with other Δ5 steroids. It has to be stressed that in the experiments described in this paper the medium was just pipetted off the cells and no efforts were made to remove the steroids inside the cells (such as rinsing the dishes with hexane). Apparently, large amounts of intermediate steroids were excreted, especially by the human Leydig cells, with P5 and 17OHP5 being the most remarkable. These data are in conflict with the idea of organized enzyme complexes [23] that keep all intermediates tightly bound to the enzymes, since according to this view 17OHP5 would not be released to the culture medium.

It has to be noted that the isolation technique used in this paper always failed when testicular tissue of prostatic carcinoma patients who had received estrogen or anti-androgen therapy was used: very low numbers of isolated cells that were almost non-responsive to hCG were obtained in the most fortunate cases (data not shown).

Comparison of the steroidogenic output of the Leydig cell cultures reveals that large variations existed between the animals tested. This was also found in other experiments performed in our laboratory using

human or rat Leydig cell cultures (data not shown). For human testes this variability recently has been published by Simpson *et al* [7] as well, but for rat Leydig cell cultures much better reproducibilities have been reported [17-19]. The reason is unclear, but stress may have played a role. In 4 experiments in which rats were taken out of their daily cages and were kept in a plastic bucket for a couple of hours before sacrifice, the basal and hCG-stimulated Ti production was considerably lower than in parallel experiments wherein the animals were sacrificed almost immediately (data not shown). This factor might play a role in the human cultures as well, apart from the differences in age or life patterns of the patients tested.

In conclusion, a simple technique for the isolation and culture of highly responsive human Leydig cells was developed. Confirming earlier data obtained in testicular homogenates, the results presented in this paper for the first time indicate that in human cultured Leydig cells the Δ5 biosynthetic pathway leading from P5 to T is the most important, whereas in the rat cells the Δ4 pathway prevails.

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HUMAN LEYDIG CELLS IN CULTURE. II. DEVELOPMENT OF TECHNIQUES FOR THE STUDY OF MITOCHONDRIAL AND MICROSOMAL STEROIDOGENIC PROCESSES.

ABSTRACT

In a previous paper we studied the steroidogenic response to hCG of cultured human and rat Leydig cells. In this paper we present additional techniques that enable separate study of the mitochondrial and microsomal steroidogenic processes of cultured Leydig cells using specific enzyme inhibitors. The use of SU-10603 and epostane, inhibitors of pregnenolone (P5) metabolism, allows study of the cholesterol side-chain cleavage (CSCC) activity by determination of P5 production via a direct RIA procedure. Combined use of [^{14}C]P5 and the CSCC inhibitor aminoglutethimide enables study of the microsomal enzyme system without interference of the results by endogenous P5 production. In addition, the data obtained in this paper in cultured Leydig cells confirm results reported earlier for testicular homogenates with respect to the preference of Δ4 or Δ5 steroids and the presence or absence of specific enzyme activities, such as 5α-reductase and 16-ene-synthetase. For the first time, the latter enzyme, which is responsible for the synthesis of (precursors of) the human sex phe omones, was found to be present in human but not in rat Leydig cell cultures.

In preparation for J Androl.

INTRODUCTION

In a previous paper [1] we described the isolation and culture of human and rat Leydig cells and we studied the steroidogenic response to hCG. In this paper we present techniques that enable the separate study of the mitochondrial and microsomal steroidogenic processes.

Numerous compounds exert their stimulatory or inhibitory effects on testicular steroidogenesis at the level of the cholesterol side-chain cleavage (CSCC). The product of the CSCC is pregnenolone (P5), which is further metabolized by the microsomal enzymes to other steroids. A possible way to obtain information concerning the activity of the CSCC is determination of the total production of all important steroids by radioimmunoassay after extensive purification. Much easier, however, would be the determination of P5 production in the presence of inhibitors of P5 metabolism. Obviously, these inhibitors must not interfere with the CSCC and must allow a direct RIA procedure (low cross-reactivity with P5 antiserum).

To study the effects of hormones or other compounds on the microsomal enzyme systems in cultured Leydig cells essentially the same technique as we used in testicular homogenates with [4-^{14}C]P5 as substrate [2,3] (*vide infra*) can be used. In order to avoid interference of the results by endogeneous P5 production, that might be enhanced or reduced by the compound of interest, an inhibitor of the CSCC without microsomal effects must be used.

The compound SU-10603 (SU) is known to inhibit several cytochrome P450 (cyt.P450) linked oxidations [4-6] whereas the structurally closely related compounds trilostane (TRIL), epostane (EPOS) and cyanoketone (CK) [7-13] are inhibitors of both 3ß-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase. EPOS and TRIL were found to be without effects on the CSCC in human placenta [13]. CK and SU, in concentrations of 5 µM and 20 µM, respectively, are currently used in studies on CSCC in cultured rat Leydig cells [14-16]. The compound aminoglutethimide (AGI) is a well-known CSCC inhibitor with little or no effects on the testicular microsomal steroidogenic enzymes [4,17-20]. In this paper the effects of EPOS, TRIL, SU and AGI on the microsomal enzyme system are studied, first in testicular homogenates and later also in cultured human and rat Leydig cells.

MATERIALS AND METHODS

testis tissue

Human testis tissues were obtained from patients who underwent subcapsular orchiectomy for their prostatic carcinoma. None of them recieved medication known to interfere with steroidogenesis. Rat testes were obtained from adult Wistar rats weighing about 200 gr killed by cervical dislocation. Testis tissue was transferred to 0.9% NaCl and used immediately.

chemicals

SU (SU-10603, 7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphtalenone) was kindly donated by C.A. Brownly Jr (Ciba Geigy, Summit, New Jersey). The compounds TRIL (trilostane, win-24540, 2α-cyano-4α,5α-epoxy-androstan-17β-ol-3-one) and EPOS (epostane, win-32729, 2α-cyano-4α,5α-epoxy-4β,17α-dimethylandrostan-17β-ol-3-one) were gifts from D.O. Sanders (Sterling Research Group Europe, Sterling Winthrop, Haarlem, The Netherlands). AGI (aminoglutethimide, 3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione) was purchased from Sigma (St. Louis, MO), hCG (pregnyl) from Organon (Oss, The Netherlands) and other chemicals from several commercial suppliers.

the metabolism of [^{14}C]P5 in testicular homogenates

The techniques of tissue homogenization, incubation and analysis of the metabolites has been described in detail elsewhere [2,3]. In short, testis tissue was homogenized on ice in 100 mM phosphate buffer pH 7.4 containing 0.25 M sucrose and centrifuged for 20 min at 10^4xg at 4°C. The supernatant was diluted with 50 mM phosphate buffer pH 7.4 to about 3 ml/gram testis tissue. Incubation of 100 µl homogenate with about 0.1 µg [4-^{14}C]P5 in a final volume of 1 ml was performed in air at 32°C in the presence of the inhibitors mentioned at the desired concentrations, NAD (final concentration, 0.4 mM) and a NADPH-generating system (final concentrations, 0.4 mM NADP, 4 mM glucose-6-phosphate, and 0.12 U/ml glucose-6-phosphate dehydrogenase). The reaction was terminated by adding ice-cold diethylether. Tritiated

marker steroids (P5, 17OHP5, DHEA, A5, P4, 17OHP4, A4, T, and estradiol) were added to monitor procedural losses. The incubation mixtures were extracted twice with diethylether and analyzed by HPLC using a diol column (Hibar LiChrosorb diol, Merck, Rahway, NJ; 5 µm) with a n-hexane/isopropanol gradient, enabling the separation of a large number of steroids in a single run [2,3]. The eluate was fractionated in 154 vials (3/min) and assayed for ^{3}H and ^{14}C in a liquid scintillation counter.

Isolation and culture of Leydig cells

Human and rat Leydig cells were isolated and cultured as described previously [1]. In short, rat testes were carefully decapsulated without cutting or teasing the tissue whereas human testes were cut in little pieces. Subsequently it was treated with collagenase in a shaking water bath at 32°C. After filtration of the cell suspension through 60 µm nylon gauze the cells were washed with culture medium (MEM containing penicillin, streptomycin, fungizone and amino acids) and were finally resuspended in culture medium containing 1% fetal calf serum. About 10^{6} nucleated cells were preincubated during 1 (rat) or 2 (man) hr in a humidified atmosphere (95% air, 5% CO_2, 32°C). The floating cells and the medium were removed by suction and the cells attached to the dishes were washed with culture medium and finally incubated for 2 (rat) or 3 (man) hr (95% air, 5% CO_2, 32°C) with serum free culture medium containing the additions mentioned in the text. The medium was removed and stored at -20°C prior to analysis.

radioimmunoassay (RIA)

Steroid production by cultured Leydig cells was determined by RIA. The techniques have been described in detail elsewhere [1,21-23]. In short, the procedure was as follows. If chromatographic purification of the steroids was necessary, [^{3}H]marker steroids (about 10^{4} dpm) were added to the samples. They were subsequently extracted with diethylether and the steroids were separated using the HPLC system as described above [1]. Aliquots of the purified steroids were used to determine the recoveries. The samples and standard curves in duplo were incubated with [^{3}H]steroid and antiserum overnight at 4°C.

Separation of the bound and free fraction was performed using dextran coated charcoal. An aliquot of the antiserum-bound fraction was assayed for ^{3}H in a liquid scintillation counter. If necessary, the concentrations were corrected for the extra amount of recovery tracer added to the samples.

RESULTS

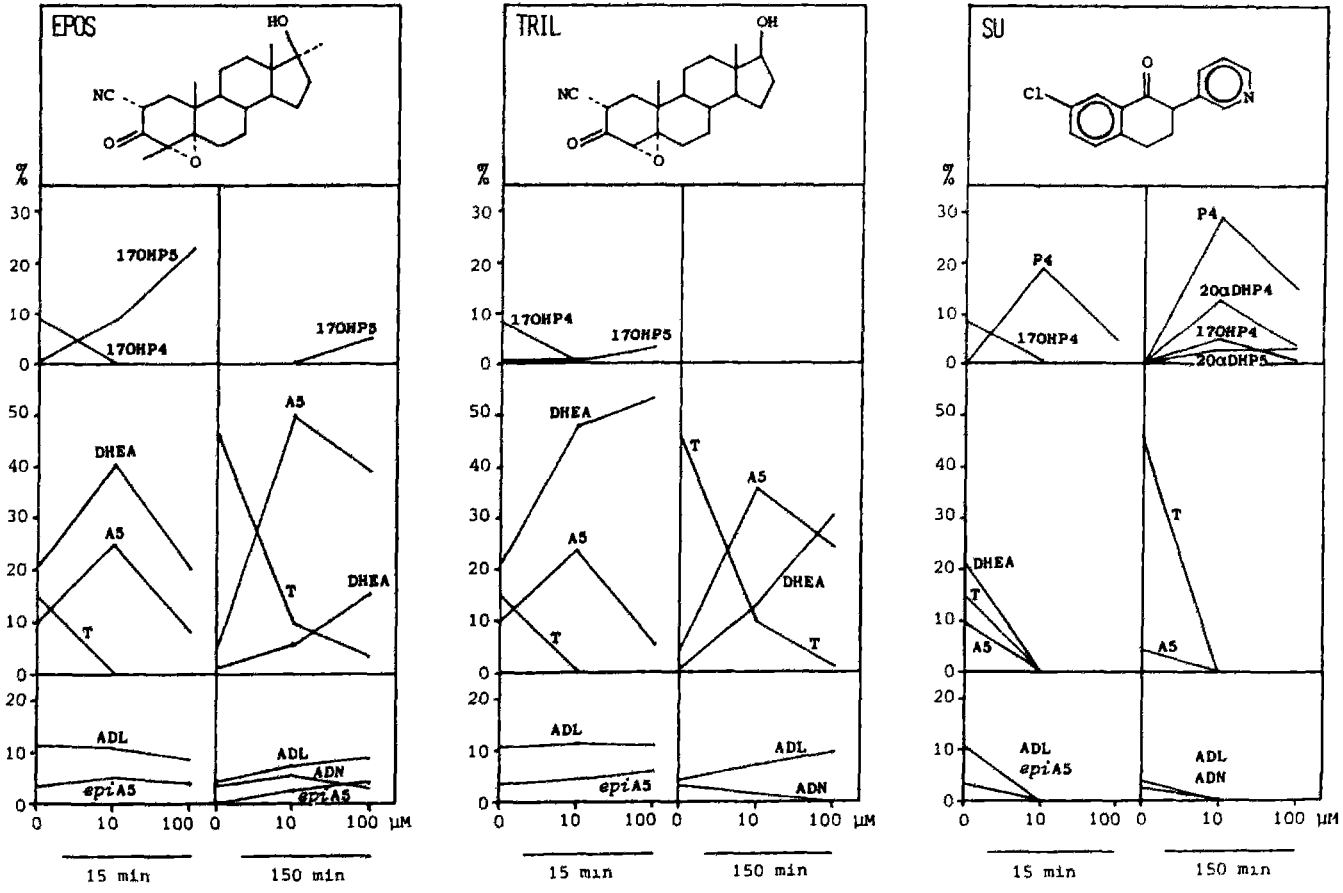
the effects of TRIL, EPOS, SU and AGI on the metabolism of [^{14}C]P5 in human testicular homogenates

In fig 1 the effects of TRIL, EPOS and SU on the microsomal enzyme system are presented (1 typical example of 4 experiments shown). The structurally closely related compounds EPOS and TRIL strongly inhibited the 3βHSD reaction as the yields of Δ4 steroids (17OHP4, T) dramatically decreased after addition of these compounds. Interestingly, most 3βHSD catalyzed reactions were inhibited approximately equally by EPOS and TRIL, the sole exception being the conversion of ADL to ADN which was virtually unaffected by 10 μM EPOS. At 100 μM EPOS inhibited the lyase reaction to some extent as well, reflected by the accumulation of 17OHP5 over DHEA and A5 (especially after 15 min of incubation). SU strongly inhibited all microsomal cyt.P450 linked reactions as P4 was the only quantitatively important metabolite of [^{14}C]P5 formed after 15 min of incubation. Remarkably, after 150 min the 20α-hydroxylated C_{21} steroids 20αOHP4 and 20αOHP5 were found. The CSCC inhibitor AGI had no effects (data not shown).

inhibition of the microsomal enzyme system in cultured Leydig cells

Human Leydig cells from a 61 yr old man were cultured in the presence of microsomal inhibitors and 0.3 IU/ml hCG as indicated in table 1. In the culture media the concentrations of the 8 steroids of the Δ4 and Δ5 pathway (P5, 17OHP5, DHEA, A5, P4, 17OHP4, A4 and T) were measured by RIA after purification via HPLC. The concentrations of the 16-androstenes ADL and ADN [24,25] could not be determined since no tracers and antisera were available. The results indicate that in the presence of 20 μM SU and 5 μM TRIL or EPOS the hCG-induced rise in total steroidogenic output almost completely consisted of P5,

Fig 1. The effects of addition of EPOS, TRIL and SU on the metabolism of [^{14}C]P5 in human testicular homogenates (1 example of 4 experiments shown). The effects were studied using different concentrations and incubation times.



indicating an effective inhibition of further conversion of this steroid.

Table 1. Basal and hCG stimulated steroid production of cultured human Leydig cells in the presence or absence of inhibitors of P5 metabolism (in pmol/ml). 'tot' indicates the sum of P5, 17OHP5, DHEA, A5, P4, 17OHP4, A4 and T.

inhibitors		-	SU+TRIL	SU+EPOS
basal	P5	2	4	5
	tot	6	6	7
hCG	P5	4	37	38
	tot	34	39	41
rise	P5	2	33	33
	tot	28	33	34

Rat Leydig cells were incubated with 0 or 1 IU/ml hCG in the presence or absence of 5 μM EPOS and 20 μM SU. The production of P5-like and T-like immunoreactivity (P5i and Ti, respectively) was determined by a direct RIA. SU and EPOS did not cross-react with the P5 and T antisera (<0.01%). In a typical experiment, the concentration of P5i in the absence of hCG was 0.1 pmol/ml and in the presence of hCG 5.5 pmol/ml. For Ti the data were <0.05 and 0.07 pmol/ml, respectively, indicating that the conversion of P5 to T was strongly inhibited. A similar experiment was performed with TRIL in stead of EPOS, but this compound cross-reacted for 90% with the T antiserum, making the results useless.

Finally, human and rat Leydig cells (N=1 and N=3, respectively; table 2) were incubated with [^{14}C]P5 in the presence of microsomal inhibitors. Using only SU or EPOS the results obtained in human testicular homogenates with respect to the inhibitory effects of these compounds were confirmed in rat Leydig cell cultures. In the presence of SU high yields of P4 and less of its 5α-reduced equivalent were found in rats and little or no other metabolites; 17αOHase activity was strongly inhibited. In the presence of EPOS high yields of numerous Δ5 steroids were found and virtually no Δ4 or 5α-reduced steroids, indicating an efficient inhibition of 3βHSD. When SU and EPOS were used simultaneously the metabolism of [^{14}C]P5 was inhibited almost

Table 2a. The effects of addition of 20 µM SU and 5 µM EPOS or TRIL on the metabolism of [^{14}C]P5 by cultured rat Leydig cells. Yields are expressed as percentage of total amount of radioactivity added as [^{14}C]P5. 0, yield <0.5%; -, undetectable.

inhibitors	rat 1				rat 2			rat 3	
	-	SU	EPOS	SU EPOS	-	SU TRIL	SU EPOS	-	SU EPOS
P5	34	42	41	97	30	99	100	13	97
17OHP5	5	1	29	0	5	0	-	5	2
DHEA	0	-	20	-	-	-	-	-	-
A5	1	-	8	-	-	-	-	-	-
P4	3	51	-	0	10	0	-	5	-
17OHP4	8	-	-	-	14	-	-	11	-
A4	20	-	-	-	16	-	-	25	-
T	27	-	-	-	9	-	-	27	-
5αP4	-	4	-	-	1	-	-	-	-
5α17OHP4	-	-	-	-	2	-	-	-	-
5αA4	-	-	-	-	4	-	-	-	-
DHT	1	-	-	-	6	-	-	4	-
5αP5	-	1	-	-	-	-	-	-	-
5α17OHP5	-	-	-	-	-	-	-	2	-
5αDHEA	-	-	-	-	-	-	-	-	-
Aadiol	-	-	-	-	3	-	-	4	-

5αP4, 5α-pregnane-3,20-dione
5α17OHP4, 17α-hydroxy-5α-pregnane-3,20-dione
5αA4, 5α-androstane-3,17-dione
5αP5, 3ξ-hydroxy-5α-pregnan-20-one
5α17OHP5, 3ξ,17α-dihydroxy-5α-pregnan-20-one
5αDHEA, 3ξ-hydroxy-5α-androstan-20-one

Table 2b. The effects of addition of 20 µM SU and 5 µM EPOS on the metabolism of [^{14}C]P5 by cultured human Leydig cells (61 yr). For details see table 2a.

inhibitors	P5	17OHP5	DHEA	A5	P4	17OHP4	A4	T	ADL	ADN	epiA5
-	32	31	11	2	1	3	1	2	4	7	3
SU+EPOS	99	-	-	-	1	-	-	-	-	-	-

completely both in man and rat. In the human Leydig cells the synthesis of the 16-androstenes was completely blocked as well.

Inhibition of the CSCC

To determine the optimal concentration of AGI necessary to inhibit the CSCC in cultured rat and human Leydig cells, incubations were performed in the presence of 1 IU/ml hCG, inhibitors of the metabolism of P5 (20 µM SU and 5 µM EPOS; *vide supra*), and a varying concentration of AGI. The steroidogenic output was determined by measuring P5 by a direct RIA. The presence of AGI did not interfere with the RIA. The data of one of the 2 experiments performed on each species are presented in fig 2, and indicate that AGI, at a concentration of about 200 µM, completely reversed the stimulatory effect of hCG.

To verify the results obtained in testicular homogenates on the lack of interference of AGI with the microsomal metabolism of [^{14}C]P5 (*vide supra*), Leydig cells of a 68 yr old prostatic carcinoma patient were incubated with [^{14}C]P5 in the presence or absence of 250 µM AGI. The dishes were rinsed twice with n-hexane to remove the ^{14}C-labeled metabolites inside the cells. No differences in the pattern of metabolites formed after 3 hr of incubation were found (table 3).

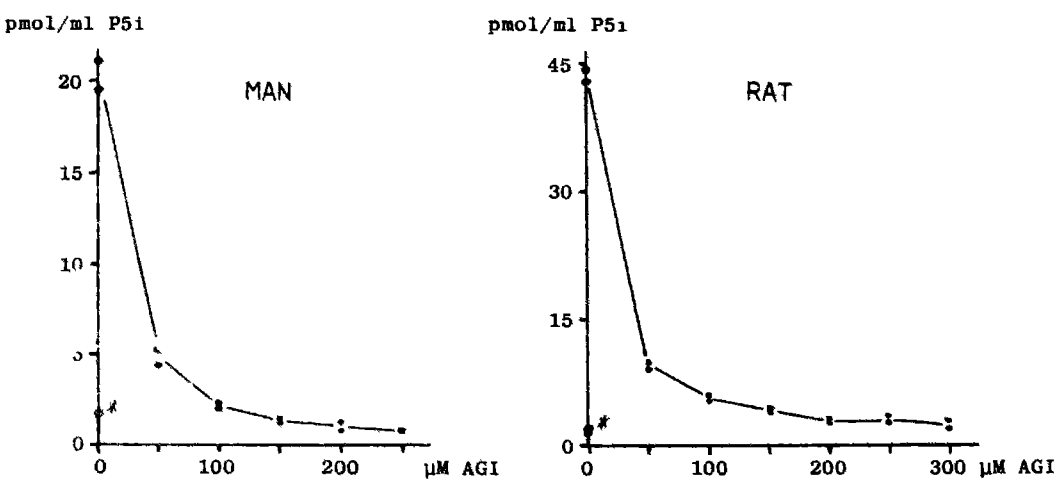


*Fig 2. Steroid production by cultured human and rat Leydig cells in the presence of 1 IU/ml hCG, 5 µM EPOS, 20 µM SU, and an increasing concentration of AGI. Steroid production was determined via a direct RIA on P5 (P5i). *: production of P5i in the absence of AGI and hCG.*

Table 3. Lack of effects of 250 μM AGI on [^{14}C]P5 metabolism by cultured Leydig cells of a 68-yr old prostatic carcinoma patient (in percentage of total radioactivity)

AGI	P5	17OHP5	DHEA	A5	P4	17OHP4	A4	T	ADL	ADN	*epi*A5
0	49	18	11	2	2	3	0	2	4	4	2
250	51	19	11	2	2	2	0	2	4	5	2

DISCUSSION

In this paper techniques for the separate study of the microsomal and mitochondrial steroidogenic processes in cultured human and rat Leydig cells are presented. The use of SU and EPOS, inhibitors of P5 metabolism, enables the determination of the CSCC activity by measuring P5 production via a direct RIA procedure. The use of [^{14}C]P5 in combination with the CSCC inhibitor AGI enables study of the microsomal enzyme system via techniques very similar to those used for the study of steroid metabolism in testicular homogenates [2]. The advantage of both procedures over determination of a plurity of steroids by RIA after chromatographic purification is more than a considerable gain in time, since for the RIA procedure a more or less arbitrary selection has to be made, resulting in an underestimation of the CSCC activity and in only a fragmentary study of the microsomal enzyme system.

The experiments using SU, TRIL and EPOS in testicular homogenates and in Leydig cell cultures indicate that these compounds are effective inhibitors of the microsomal enzyme systems. The hCG induced rise in the total steroidogenic output in the absence of inhibitors (28 pmol/ml; table 1) was about 80% of that in the presence of SU and TRIL (33 pmol/ml) or SU and EPOS (34 pmol/ml). This difference of about 20% might be explained by the presence of 16-ene-synthetase activity in the human testis, which is inhibited by SU. As a result, the total steroidogenic output in the absence of inhibitors as presented in table 1 is an underestimation, since synthesis of 16-androstenes for technical reasons could not be determined. A conversion of P5 to these steroids of about 20% is in line with the yields of ADL, ADN and other 'unidentified metabolites' found in testicular homogenates [2,3,24]. These data indicate that inhibition of the CSCC by these compounds is not very likely.

The data presented in tables 2 and 3 on the metabolism of [^{14}C]P5 by cultured Leydig cells confirm the results we earlier obtained using similar techniques in human and rat testicular homogenates [2,24]. Furthermore they confirm our recent data on the steroidogenic response to hCG in Leydig cell cultures [1]. In the human Leydig cells high amounts of Δ5 steroids were formed from [^{14}C]P5, but in the rat the Δ4 steroids were quantitatively much more important. A number of different 5α-reduced steroids were found in the rat Leydig cell cultures, but they were not formed in detectable amounts in the human cells. Measurable amounts of estrogens were not found in either species. In addition, the data for the first time indicate that in the human testis the Leydig cells are the major source of the 16-androstenes ADL and ADN, sex pheromone precursors in man, found previously in testicular homogenates [24].

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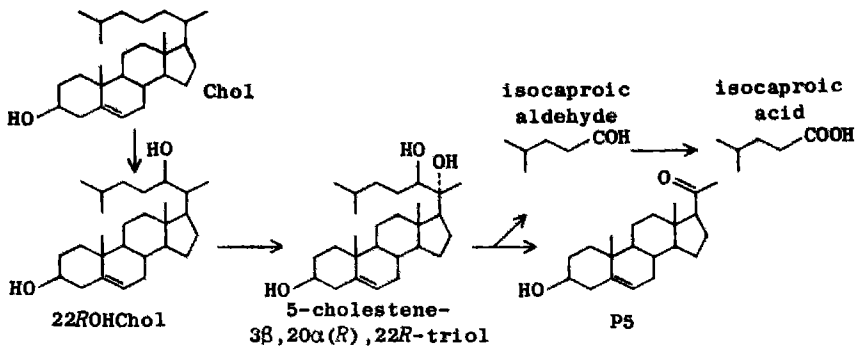
HYDROXYCHOLESTEROLS AND AMINOGLUTETHIMIDE

Chol is the precursor for almost all steroids known and is converted to P5 by the CSCC-complex in the mitochondria. Due to the lipophilic nature of this sterol several hitherto uncompletely understood mechanisms play an important role *in vivo* to supply the CSCC-complex with Chol [1-4]. The side chain cleavage itself [5-8] is initiated by hydroxylations at the carbons 20 and 22:



The intermediate sterols in this figure are approximate structures of the real intermediates that remain bound to the CSCC complex.

Hydroxycholesterols (i.e. 20αOHChol, 22*R*OHChol and 25OHChol) are frequently used to study the CSCC *in vitro* [7-9] because these sterols can reach the CSCC-complex by simple diffusion. As a result, these sterols can be added to the culture medium without addition of other essential compounds to enable the entrance in the cells.

As explained in chapter 8, the compound AGI is known to inhibit the CSCC. In order to study the level of inhibition the following experiment was performed.

The effect by the addition of 200 μM AGI on P5 production of rat Leydig cells in the presence of 80 μM of the hydroxycholesterol indicated and inhibitors of P5 metabolism (20 μM SU and 5 μM EPOS; chapter 8) was studied (N=2). P5-production was determined by RIA after purification of the samples via paper chromatography using the modified Bush-A solvent system [10,11]. A direct RIA procedure was not possible since the hydroxycholesterols cross-reacted with the P5 antiserum used. Unfortunately, 20αOHChol was not separated from P5 in the paper

system used, so only the results of the other two hydroxycholesterols could be analyzed. The ratio of 22*R*OHChol-mediated steroid production in the presence of AGI over that in its absence was 0.45 ± 0.03, indicating only a 55% inhibition of the conversion of 22*R*OHChol to P5 by AGI. For 25OHChol the ratio was 0.034 ± 0.002, indicating a 97% inhibition. Assuming that 25OHChol is converted to P5 via essentially the same process as Chol (*vide supra*), i.e. via the 25-hydroxylated derivate of 22*R*OHChol, and that the inhibition of the conversion of the latter intermediate to P5 is the same as that of 22*R*OHChol to P5 (55%), an estimation of the inhibition of the 22-hydroxylation of 25OHChol can be calculated. Of the 1000 molecules of Chol only 34 (ratio 0.034) are converted to P5 in the presence of AGI. These 34 molecules represent 45% of the total amount of 22*R*OHChol formed, indicating that about 76 molecules 22*R*OHChol were formed from Chol and consequently that the 22-hydroxylation was inhibited about 92%. These data strongly suggest that in cultured rat Leydig cells AGI exerts its inhibitory effect on the CSCC mainly on the level of the first hydroxylation, i.e. at C-22. These data also indicate that 25OHChol is a more physiological model for Chol than 22*R*OHChol in the study of the CSCC, since 22*R*OHChol bypasses a considerable part of the CSCC process, whereas 25OHChol does not.

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TIME SEQUENCE IN [^{14}C]PREGNENOLONE METABOLISM IN CULTURED LEYDIG CELLS

The use of [^{14}C]P5 in Leydig cell cultures, as described in chapter 8, also enabled the study of the time sequence of the metabolism of this steroid. Since [^{14}C]P5 is metabolized *in* the cells, the dishes were rinsed twice with n-hexane to remove the intracellular ^{14}C-labeled metabolites. The results of the time sequence studies are presented in fig 1.

Comparison of the profiles obtained in cultured rat and human Leydig cells with those of testicular homogenates reveals that, apart from the reaction rates, virtually no differences were found with respect to the preferred pathways leading from P5 to T and the occurrence of 'unidentified metabolites'. In rat Leydig cells the Δ4 pathway via P4 was the preferred one with 17OHP5 as the only quantitatively important Δ5 metabolite. Numerous 5α-reduced steroids were formed, most of them in low yields. No estrogens or 16-androstenes were found (only 1 of 2 experiments shown).

Unfortunately, only a single experiment on the time sequence of [^{14}C]P5 in cultured human Leydig cells was performed (fig 1). In this experiment the reaction rates were very low: yield of T after 3 hr of incubation was still less than 0.5% and after 8 hr only 2%. It is possible that the age of the patient (85 yr) was partially debet to these low reaction rates since in other experiments using Leydig cells from a 61 and 68 yr old man and a single incubation time of 3 hr clearly higher reaction rates were found as considerable greater amounts of [^{14}C]P5 were metabolized (table 1). Interestingly, the fraction of [^{14}C]P5 that remained unmetabolized during the 3 hr of incubation increased with the age of the 3 patients tested. As can be derived from table 1, the yields of P4 expressed as percentage of the total amount of metabolites formed from [^{14}C]P5 differed markedly between the patients tested and was the highest (i.e. 17%) in the patient of fig 1. In the other patients the data were 1.5% (61 yr) and 4% (68 yr). In spite of these differences, the data obtained in the time sequence study confirm the results obtained in testicular homogenates, since [^{14}C]P5 was metabolized preferentially via the Δ5 pathway to DHEA. As has already been remarked in chapter 8, the appearance of ADL and ADN reflect the presence of 16-ene-synthetase

also in cultured human Leydig cells. No 5α-reduced steroids or estrogens were formed in the incubations.

Fig 1. Time sequence in [^{14}C]P5 metabolism in cultured Leydig cells

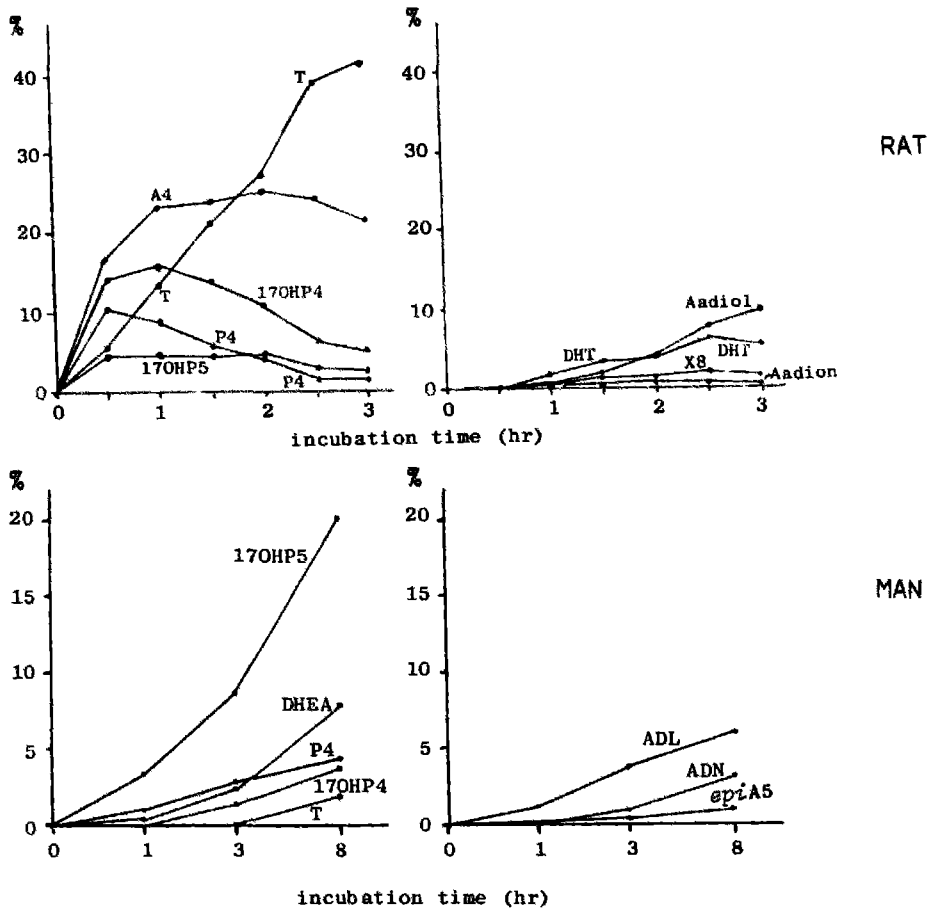
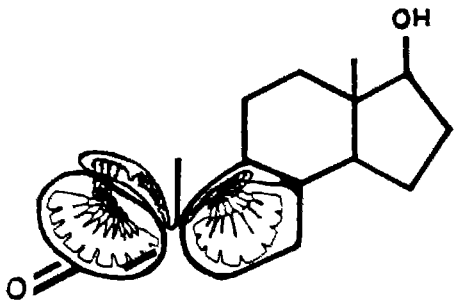


Table 1. The patterns of metabolites formed from [^{14}C]P5 by cultured Leydig cells of three patients after 3 hr of incubation (in percents of total radioactivity)

age	P5	17OHP5	DHEA	A5	P4	17OHP4	A4	T	ADL	ADN	*epi*A5
61	32	31	11	2	1	3	1	2	4	7	3
68	49	18	11	2	2	3	0	2	4	4	2
85	77	9	3	0	4	1	0	0	4	1	1

miscellaneous



part IV

In chapters 2, 3, and 4 of this thesis experiments using testicular homogenates of rats, monkeys and men have been described and discussed. The data indicated that large differences in [^{14}C]P5 metabolism were found between the species tested. In the rat T was rapidly synthesized from P5 almost completely via the Δ4 steroid 17OHP4 that was formed via both P4 and 17OHP5. In the monkeys (crab eating monkeys and rhesus monkey) P5 metabolism was rather slow, whereas the Δ4 pathway via P4 was the preferred route. In the human testes the Δ5 pathway via A5 was predominant. Furthermore, profound differences were found between the species in the total pattern of metabolites formed from [^{14}C]P5 or other [^{3}H]substrates. Only in the rat testicular homogenates 5αRed activity was present, whereas in the monkey and human testicular homogenates 20αHSD and 16αOHase activities were found. Only in the human testes 16-ene-synthetase activity was present, resulting in synthesis of the sex pheromone precursors ADL and ADN (X1).

The study of steroid metabolism in testicular homogenates precluded observations on the CSCC and several receptor and 2nd messenger systems. To enable this kind of studies a technique for the isolation and culture of Leydig cells was developed. In chapters 7 and 8 of this thesis several experiments using cultured human and rat Leydig cells are described and discussed. The results obtained in homogenates and cultures were in complete agreement with each other. Once again, the predominance of Δ4 steroids in Leydig cells of rats and of Δ5 steroids in humans was found. The presence of 5αRed activity in rat testicular homogenates was confirmed in the culture experiments, just as the absence of this enzyme in human testes and the absence of measurable aromatase activity in both species. The 16-androstenes were synthesized in the human but not in the rat Leydig cells. From the foregoing it can be concluded that the use of homogenates does yield reliable information concerning the preferred pathways and presence or absence of specific enzyme activities.

Comparing the advantages and disadvantages of homogenate and culture studies with each other, the following considerations have to be made. The homogenate technique *per se* is easier and - at least so far - more reliable than the culture technique. A major advantage of the study of cultured Leydig cells is that receptor-mediated processes can be studied, which is obviously impossible in homogenates. As

discussed above, the results obtained in homogenates and cultures with respect to several enzyme characteristics are in complete agreement. It has to be noted that a biopsy specimen with a weight of about 50 mg already enables study via the homogenate technique, but is too small to enable isolation of Leydig cells, making the homogenate technique a useful diagnostic tool in the assessment of testicular steroidogenic disorders. Maybe in the future the culture technique will be improved in such a way that both techniques can be used complementary to each other.

SUMMARY

In this thesis the experiments performed on the metabolism and synthesis of steroids by testicular tissue of humans, monkeys and rats are described and discussed. Chapters 2, 3, and 4 (part IIa) deal with time sequence studies of [^{14}C]pregnenolone ([^{14}C]P5) metabolism in rat, human, and monkey testicular homogenates, chapters 5 and 6 (part IIb) with enzymatic conversion studies, and chapters 7 and 8 (part III) with studies on Leydig cell cultures.

In chapter 2 the technique developed for the study of the *in vitro* metabolism of radiolabeled steroids, especially [4-^{14}C]P5, in testicular homogenates is described. This technique involves the incubation of a testicular homogenate with [^{14}C]P5 or another appropriate radiolabeled steroid in the presence of NAD and a NADPH generating system. If [^{14}C]labeled substrate was used, 9 [^{3}H]marker steroids were added after termination of the reaction. The mixture was analyzed via HPLC enabling the separation of a large number of steroids in a single run. The data obtained in rat, monkey and human (prostatic carcinoma) testicular homogenates (chapters 2 and 4) indicated that, under the conditions used, large differences existed between the species with respect to the conversion rates, the preferred pathways leading from P5 to testosterone (T) and the presence of other enzymatic activities. In the rat testes T was synthesized rapidly (within 1 min) from [^{14}C]P5 almost exclusively via 17α-hydroxyprogesterone (17OHP4), that was formed via both progesterone (P4) and 17α-hydroxypregnenolone (17OHP5). In the monkeys (crab eating monkey, *Macaca irus*, and rhesus monkey, *Macaca mulatta*) P5 metabolism proceeded relatively slow and the Δ4 pathway via P4 was the preferred one; low or undetectable levels of any Δ5 metabolite were found. As compared to rats, in the human testes P5 metabolism was also rather slow, whereas the Δ5 pathway via dehydroepiandrosterone (DHEA) and androstenediol (A5) appeared to be the preferred pathway with 17OHP4 and T as the only quantitatively more or less important Δ4 metabolites. Levels of P4 and androstenedione (A4) remained low or undetectable.

Apart from intermediates in the synthesis of T several other metabolites were formed from [^{14}C]P5 under the conditions used. These metabolites are referred to as 'unidentified metabolites', designated X1 to X10. The experiments that were performed to characterize these metabolites are described in chapter 3 and addendum E. As expected, in

the rat testicular homogenates several 5α-reduced steroids were formed (5α-dihydrotestosterone (DHT; X3), androstanediols (Aadiol; X6), and 3ξ,17α-dihydroxy-5α-pregnan-20-one (X8)). These steroids were not found in the monkey or human testicular homogenates. In the testicular homogenates of the monkeys tested high levels of the 20α-hydroxylated steroids 20α-hydroxy-4-pregnen-3-one (20αOHP4; X4) and 17α,20α-dihydroxy-4-pregnen-3-one (17,20αP4; X9) and, to a lesser extent, the 16α-hydroxylated steroid 16α-hydroxyprogesterone (16αOHP4; X10) were formed from [^{14}C]P5. Due to the low amounts of P4 formed in the human testicular homogenates, the presence of 20α-hydroxysteroid dehydrogenase and 16α-hydroxylase activities was hardly detectable using [^{14}C]P5 as substrate, but clearly when [^{3}H]P4 (or [^{3}H]17OHP4) was used.

The most intriguing 'unidentified metabolite' synthesized from [^{14}C]P5 was X2. Within 1 minute of incubation it was formed in high amounts in the human but not in the rat or monkey testicular homogenates. It was identified as the sex pheromone precursor 5,16-androstadien-3β-ol (ADL) using mass spectrometry as final evidence (chapter 3) and later synthesized from DHEA (addendum D). The Δ4 equivalent of ADL, 4,16-androstadien-3-one (ADN; X1), was found as well, in addition to 5-androstene-3β,17α-diol (*epi*A5; X7), the satellite of ADL. The synthesis of large amounts of the 16-androstenes ADL and ADN (max 20-25%) was rather unexpected. The 16-androstenes are well-known in pigs and amongst others responsible for the 'boar taint' that can be detected in meat of uncastrated male pigs. The occurrence and physiological role of these steroids and their 5α-reduced equivalents, the sex pheromones androstenone and androstenol, are discussed in detail in addendum C.

Aromatase activity was not measurable in any testicular homogenate studied in this thesis, but when [^{3}H]T was used as substrate in the homogenate of a full-term human placenta high yields of estradiol (E2) were obtained. Probably the conversion rate of T to E2 in the testis is too low to allow detection of the minute amounts of estrogens formed.

As already alluded to above, 5α-reductase activity was not detectable in human testicular homogenates. This was rather remarkable in the light of the presence of 5α-reduced steroids in human spermatic vein blood. This discrepancy is discussed in addendum B.

In chapter 5 the conversion of P5 to ADL, catalyzed by 16-ene-syn-

thetase, was studied in detail and a proposal for the mechanism underlying this reaction was formulated. This proposal explains our finding of a lack of intermediates in the conversion of P5 to ADL and also explains why ADL and *epi*A5, at least in our hands, always appeared together: according to our hypothesis, ADL and *epi*A5 are synthesized from P5 via two competing single-step mechanisms.

In addendum F our own results as well as literature data indicating that 17α-hydroxylase (17αOHase) and lyase activities have different properties *in vivo* as well as *in vitro* are discussed in the light of the generally accepted concept of 17αOHase and lyase being two activities associated with the same protein. The conclusion is that the ultimate activity of this cytochrome P450-linked enzyme, that might be able to exert 16-ene-synthetase activity as well, is probably not solely determined by the primary structure of the protein.

In chapter 6 the Δ5-3-ketosteroid isomerase reaction, catalyzing the 2nd step in the conversion of Δ5 steroids to their Δ4 equivalents, was studied. The results indicate that in testicular homogenates of rat, monkey and man this reaction is likely to proceed via essentially the same mechanism as previously reported for the micro-organism *Pseudomonas testosteroni*, i.e. via an intramolecular hydrogen shift from the 4ß- to the 6ß-position.

In chapters 7 and 8 and addenda G and H experiments on Leydig cell culture are described. A simple technique for the isolation and culture of human Leydig cells was developed and the steroidogenic response to human chorionic gonadotropin (hCG) was studied in detail. Techniques for the separate study of the mitochondrial and microsomal steroidogenic processes using specific enzyme inhibitors (SU-10603, epostane, aminoglutethimide) were developed. Both human and rat Leydig cells were highly responsive to hCG, resulting in a manyfold increase in steroid production in both species. In line with the data obtained in the testicular homogenates, in the rat Leydig cell cultures high amounts of the Δ4 steroids A4 and T were formed in response to hCG, whereas in the human Leydig cell cultures the Δ5 steroid DHEA was quantitatively the most important steroid formed. Using [^{14}C]P5 in cultured Leydig cells the prevalence of Δ4 steroids in rat and of Δ5 steroids in human testes was again confirmed. These data for the first time demonstrate that the Δ5 pathway is the main pathway in the biosynthesis of T in cultured human Leydig cells. In the experiments with

[^{14}C]P5 the presence of 5α-reductase in rat, but not in human and the absence of measurable aromatase activity in human and in rat testes was found, once again confirming the results obtained in testicular homogenates. The most intriguing finding, however, was that in the human testis the Leydig cells were the major source of the 16-androstenes, sex pheromone precursors unique (so far) to pigs and human primates. No 16-ene-synthetase activity was found in rat Leydig cells.

SAMENVATTING

In dit proefschrift zijn experimenten betreffende de synthese en het metabolisme van steroiden in testisweefsel van mensen, apen en ratten beschreven en bediscussieerd. In hoofdstukken 2, 3 en 4 (deel IIa) is het onderzoek naar de tijdsafhankelijkheid van het metabolisme van ^{14}C-gemerkt pregnenolon ([^{14}C]P5) in testishomogenaten beschreven, en in hoofdstukken 5 en 6 (deel IIb) studies naar enkele reaktiemechanismen die aan de omzettingen van steroiden ten grondslag liggen. Hoofdstukken 7 en 8 (deel III) gaan over experimenten met Leydigcelcultures.

In hoofstuk 2 is de techniek welke ontwikkeld werd voor de studie van het metabolisme van steroiden, voornamelijk [^{14}C]P5, in homogenaten van testisweefsel beschreven. Deze techniek omvat de incubatie van een testishomogenaat met [^{14}C]P5 of een ander radioaktief gemerkt steroid in aanwezigheid van NAD en een NADPH genererend systeem. In de gevallen dat [^{14}C]gemerkt substraat werd gebruikt werden, na het stoppen van de reactie, 9 [^{3}H]steroiden toegevoegd. Het mengsel werd vervolgens geanalyseerd via HPLC, waarmee een groot aantal steroiden in een enkele run van elkaar kon worden gescheiden. De resultaten verkregen in testishomogenaten van ratten, apen en mensen (prostaatcarcinoompatiënten) (hoofdstukken 2 en 4) maakten duidelijk dat er, onder de gebruikte omstandigheden, grote verschillen bestaan tussen de bestudeerde soorten, zowel wat betreft de reaktiesnelheden, het verloop van de omzettingen van P5 naar testosteron (T), als de aan- of afwezigheid van andere enzymen. In de testishomogenaten van de rat werd T zeer snel (binnen 1 min) uit [^{14}C]P5 gevormd en vrijwel uitsluitend via 17α-hydroxyprogesteron (17OHP4), dat op zijn beurt zowel via progesteron (P4) als via 17α-hydroxypregnenolon (17OHP5) werd gesynthetiseerd. In de testishomogenaten van 2 soorten apen (Java-aap, *Macaca irus*, en rhesus aap, *Macaca mulatta*) verliep het metabolisme relatief langzaam en had de Δ^4-route via P4 de voorkeur; er werden geen of slechts geringe hoeveelheden Δ5 steroiden gevormd. In vergelijking met het metabolisme in ratte-testes verliep het metabolisme van P5 in de humane testes vrij langzaam. De synthese van T verliep vrijwel uitsluitend via de Δ5 steroiden dehydroepiandrosteron (DHEA) en androsteendiol (A5). Van de Δ4 steroiden werden alleen T en 17OHP4 in kwantitatief min of meer belangrijke hoeveelheden gevormd terwijl de synthese van P4 en androsteendion (A4) laag of ondetecteerbaar

bleef.

Behalve tussenprodukten in de synthese van T werden uitgaande van [^{14}C]P5 nog een aantal andere metabolieten gevormd. Ze werden aangeduid met de term 'ongeïdentificeerde metabolieten', genummerd X1 tot X10. De experimenten die werden verricht om deze metabolieten te karakteriseren zijn beschreven in hoofdstuk 3 en addendum E. Volgens verwachting werden in de testishomogenaten van ratten een aantal 5α-gereduceerde steroïden gevonden (5α-dihydrotestosteron (DHT; X3), androstaandiolen (Aadiol; X6) en 3ξ,17α-dihydroxy-5α-pregnaan-20-on (X8)). Deze steroïden werden niet gevonden in testishomogenaten van apen en mensen. In de testishomogenaten van de bestudeerde apen werden uitgaande van [^{14}C]P5, naast het 16α-gehydroxyleerde steroïd 16α-hydroxyprogesteron (16αOHP4; X10), grote hoeveelheden van de 20α-gehydroxyleerde steroïden 20α-dihydroprogesteron (20αDHP4; X4) en 17α,20α-dihydroxy-4-pregneen-3-on (17,20αP4; X9) gevonden. Omdat in de humane testishomogenaten in het algemeen zeer weinig of geen P4 gevormd werd uit [^{14}C]P5 werden deze steroïden bij de mens in die experimenten niet gevonden, maar bij gebruik van [^{3}H]P4 (of [^{3}H]17OHP4) als substraat was de aanwezigheid van 20α-hydroxysteroïd dehydrogenase en 16α-hydroxylase aktiviteit duidelijk meetbaar.

De meest intrigerende 'ongeïdentificeerde metaboliet' was X2. Deze werd in grote hoeveelheden in de humane testishomogenaten gevormd, maar werd in de testishomogenaten van de apen en ratten niet in meetbare hoeveelheden aangetroffen. De metaboliet werd geïdentificeerd als 5,16-androstadieen-3β-ol (ADL), een voorloper van de sex-feromonen, waarbij massaspektrometrie als uiteindelijk struktuurbewijs gebruikt werd (hoofdstuk 3). Later kon ADL worden gesynthetiseerd uitgaande van DHEA (addendum D). De synthese van het Δ4-equivalent van ADL, 4,16-androstadieen-3-on (ADN; X1), kon eveneens in de humane testes worden aangetoond, evenals de synthese van 5-androsteen-3β,17α-diol (*epi*A5; X7), de satelliet van ADL. De vorming van grote hoeveelheden van de 16-androstenen ADL en ADN door de humane testes (max 20-25%) was onverwacht. Deze groep steroïden was alleen goed bekend bij varkens en ondermeer verantwoordelijk voor de 'beregeur' van vlees van ongecastreerde mannelijke dieren. Het voorkomen en de fysiologische rol van deze steroïden en hun 5α-gereduceerde equivalenten, de sex-feromonen androstenon en androstenol, is onderwerp van addendum C.

Aromatase-aktiviteit werd niet gevonden in enig testishomogenaat

dat bestudeerd werd in dit proefschrift. Bij gebruikmaking van een homogenaat van een normale humane placenta met [^{3}H]T als substraat werd dit enzym wel gemeten. Waarschijnlijk is de aktiviteit van het aromatase-enzym in de testishomogenaten te laag om detecteerbare hoeveelheden oestrogenen op te leveren.

Zoals boven vermeld werd geen 5α-reductase aktiviteit in de humane testishomogenaten gevonden. Dit is een opvallende bevinding gezien het feit dat relatief hoge spiegels van 5α-gereduceerde steroïden in menselijk *vena spermatica*-bloed gevonden zijn. Dit probleem is onderwerp van addendum B.

In hoofdstuk 5 werd de omzetting van P5 naar ADL, gekatalyseerd door het 16-een-synthetase, in detail bestudeerd en werd een voorstel geformuleerd voor het reaktiemechanisme dat aan deze omzetting ten grondslag ligt. Deze hypothese dient ter verklaring van het feit dat in de loop van de experimenten beschreven in dit proefschrift geen aanwijzingen gevonden konden worden voor het bestaan van intermediairen in de omzetting van P5 in ADL en ook dat, althans in onze experimenten, de synthese van ADL altijd vergezeld ging van de synthese van *epi*A5: volgens onze hypothese worden ADL en *epi*A5 uit P5 gesynthetiseerd via twee competitieve één-staps reakties.

Resultaten in dit proefschrift en literatuur-gegevens wijzen erop dat 17α-hydroxylase (17αOHase) en lyase zowel *in vivo* als *in vitro* verschillende eigenschappen bezitten. Deze gegevens worden in addendum F bediskussieerd in het licht van de alom aanvaarde opvatting dat 17αOHase en lyase twee aktiviteiten van één eiwit zijn. De conclusie is dat de uiteindelijke aktiviteit van dit cytochroom P450-gebonden enzym, dat mogelijk ook nog 16-een-synthetase aktiviteit kan hebben, waarschijnlijk niet alleen bepaald wordt door de primaire struktuur van het eiwit.

In hoofdstuk 6 werd het Δ5-3-ketosteroïd isomerase, het enzym dat de tweede stap in de omzetting van Δ5 naar Δ4-steroïden katalyseert, bestudeerd. Onze gegevens wijzen erop dat deze reaktie in de testishomogenaten van rat, aap en mens waarschijnlijk via eenzelfde mechanisme verloopt als eerder werd beschreven voor het microörganisme *Pseudomonas testosteroni*, dat wil zeggen via een intramoleculaire verhuizing van de 4ß-waterstof naar de 6ß-positie.

In hoofdstukken 7 en 8 en addenda G en H werden experimenten betreffende Leydigcelcultures beschreven. Een eenvoudige techniek voor

de isolatie en cultuur van Leydigcellen werd ontwikkeld, waarna de steroïdogene respons op humaan choriongonadotropine (hCG) in detail werd bestudeerd. Ook werden technieken ontwikkeld die, met gebruikmaking van specifieke enzym-remmers (aminoglutethimide, epostaan, SU-10603), de studie van de mitochondriële en de microsomale steroïdogene processen mogelijk maken. In de Leydigcelcultures afkomstig van zowel rat als mens kon de steroïdsynthese met behulp van hCG tot vele malen de basale produktie gestimuleerd worden. In overeenstemming met de resultaten verkregen in testishomogenaten werden in de Leydigcelcultures van de rat grote hoeveelheden van de Δ4-steroïden A4 en T gesynthetiseerd als reaktie op de toediening van hCG, terwijl in de humane cultures het Δ5-steroïd DHEA kwantitatief het belangrijkste was. Gebruikmakend van [^{14}C]P5 in de celcultures kon de voorkeur voor Δ4-steroïden bij de rat en voor Δ5-steroïden bij de mens bevestigd worden. Deze experimenten tonen voor het eerst aan dat ook in cultures van humane Leydigcellen T voornamelijk via de Δ5-route gesynthetiseerd wordt. Ze bevestigen eveneens de in testishomogenaten reeds eerder aangetoonde aanwezigheid van 5α-reductase bij de rat, de afwezigheid ervan bij de mens, en bovendien het ontbreken van meetbare aromatase aktiviteit bij beide soorten. Zeer interessant is de bevinding dat in de humane testis de Leydigcellen verantwoordelijk zijn voor de synthese van de 16-androstenen, sex-feromonen, die - voor zover bekend - alleen bij de mens en het varken voorkomen. Bij de rat werd geen 16-een-synthetase aktiviteit gevonden.

DANKWOORD

Dit proefschrift was nooit tot stand gekomen zonder de hulp die ik tijdens mijn promotieonderzoek van velen heb mogen ervaren. Langs deze weg bedank ik hen allen daar van harte voor. Enkele personen wil ik echter graag speciaal noemen.

Allereerst geldt mijn dank de patienten die geheel belangeloos testisweefsel ter beschikking hebben gesteld en het medisch personeel dat hierbij als intermediair tussen hen en mij heeft willen functioneren (van het St. Radboudziekenhuis de afdelingen Urologie, Kindergeneeskunde en Obstetrie en Gynaecologie; van de afdeling Urologie van het Canisius-Wilhelminaziekenhuis Dr. H.F.M. Karthaus en Dr. R.L.F.M. Corten; van de afdeling Pathologie van het St. Maartensgasthuis te Venlo Dr. R.J.M. Retera; en van het Gemeentezickenhuis te Arnhem Dr. J.-P.A. Nicolai). De medewerkers van het dierenlaboratorium (Hoofd: Prof. Dr. W.J.J. van der Gulden) dank ik voor hun medewerking bij het verkrijgen van testikels van ratten en apen.

Veel heb ik te danken aan de afdeling Biochemie II van de Erasmus Universiteit van Rotterdam, speciaal in de persoon van Dr. F.F.G. Rommerts.

Dr. J.B. Hussem, Dr. G.H.J. Schmeits, en Dr. P.L. Jacobs (AKZO research laboratorium, Arnhem, en Organon International, Oss) dank ik voor hun enthousiaste medewerking met betrekking tot het verkrijgen van het massaspektrum zoals beschreven in hoofdstuk 3.

Drs. Gert Legemaat ben ik zeer erkentelijk voor zijn belangrijke bijdrage aan hoofdstuk 5.

Uiteraard dank ik ook alle (ex)medewerkers van de endocrinologische laboratoria voor hun aandeel. Heel speciaal wil ik mijn beide paranimfen Marianne van der Wouw en Gerard Pesman bedanken, die voor mij veel betekend hebben, ook buiten het kader van dit boekje.

Last but not least gaat mijn dank uit naar het rots-vaste thuisfront, speciaal naar mijn ouders, waarbij ik mijn vader nog speciaal bedank voor het ontwerpen van de omslag.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 1 juli 1962 in Boxtel geboren. Hij behaalde in mei 1980 het VWO-diploma te Amersfoort en hij begon met de scheikunde-studie aan de Rijks Universiteit van Utrecht in september van hetzelfde jaar. Het kandidaatsexamen werd cum laude behaald in september 1983 en het doctoraalexamen in mei 1985. Het hoofdvak van het doctoraalexamen, Organische Chemie der Natuurstoffen (Vakgroep Organische Chemie, bio-organische richting), studeerde hij bij Prof. Dr. C.A. Salemink en Dr. I. Ebels, en het bijvak Endocrinologie bij Prof. Dr. J.H.H. Thijssen en Dr. M.A. Blankenstein. In augustus 1985 werd hij als wetenschappelijk assistent aangesteld binnen de afdeling Endocriene ziekten (Hoofd: Prof. Dr. P.W.C. Kloppenborg), waar hij onder leiding van Prof. Dr. A.G.H. Smals en Prof. Dr. Th.J. Benraad (Hoofd afdeling Experimentele en Chemische Endocrinologie) de in dit proefschrift beschreven experimenten verrichtte.

STELLINGEN

1.

In de humane testikel verloopt de synthese van testosteron voornamelijk via androsteendiol en niet via androsteendion

dit proefschrift

2.

De opvatting dat dehydroepiandrosteron gezien moet worden als een "bijnier-steroid" moet, tenminste wat betreft de man, worden herzien

dit proefschrift

3.

Dihydrotestosteron in humaan *vena spermatica*-bloed is afkomstig uit de epididymis en niet uit de testis

dit proefschrift

4.

De hoge progesteronproduktie die door verscheidene auteurs in humane testishomogenaten uitgaande van pregnenolon is gemeten, staat mogelijk in verband met een onvolledige scheiding van progesteron en androstadienol

dit proefschrift

5.

Epitestosteron is een metaboliet van een verbinding die in de humane testis waarschijnlijk als bijprodukt in de synthese van 16-androstenen ontstaat

dit proefschrift

6.

De enzymatische aktiviteit van het cytochroom-P450$_{17\alpha}$ wordt door (een) onbekende verbinding(en) gereguleerd

dit proefschrift

7.

De "paar-vormende" werking welke van dansen uitgaat, wordt onder meer gevoed door de produktie van 16-androstenen in de testikel

8.

Het verschuiven van de puberteit naar een steeds lagere en van de menopauze naar een steeds hogere leeftijd kan onder meer verklaard worden via de anti-gonadotrope aktiviteit van de epifyse

9.

Het experimentele gegeven dat het Delta Sleep Inducing Peptide (DSIP) de N-acetyltransferase aktiviteit van de epifyse van de rat kan remmen suggereert een rol van dit peptide in de regulatie van de circadiane ritmen

Graf et al, J Neurochem, 44 (1985) 629-632

Noteborn et al, J Pineal Res, 5 (1988) 161-177

10.

De effecten van oestrogenen op het immuunsysteem bij de mens worden gedeeltelijk gemedieerd via receptoren in een nog onbekend type mononucleaire bloedcel

Weusten et al, Acta Endocrinol (Kbh), 112 (1986) 409-414

11.

De suggestie dat fosfaat-vrije wasmiddelen *goed* zijn voor het milieu is misleidend

12.

De baritonsaxofoon is in de harmoniemuziek nog steeds een ondergewaardeerd instrument

13.

Het verdient aanbeveling om, zoals reeds bij fietsen gebruikelijk is, ook geparkeerde auto's aan een ketting vast te leggen

ISBN 90-9002662-2