Reprint from

10th Cologne Workshop On Dope Analysis 7th to 12th June 1992 - Proceedings -

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
S. Rauth
(Editors)

Sport und Buch Strauß, Köln, 1993

S. RENDIC:
Metabolism of Testosterone

In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) 10th Cologne Workshop On Dope Analysis 7th to 12th June 1992. Sport und Buch Strauß, Köln, (1993) 27-46

S. Rendić

Metabolism of Testosterone

Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia

Endogenous testosterone is metabolised extensively in a number of tissues, and as metabolic products number of structurally related compounds can be formed (Fig. 1). At least 27 other steroids of different biological importance are potential metabolites and they can be farther metabolised to 548 isomeric monohydroxy derivatives (1). Additionally hydroxylated products (polyhydroxylated) have been identified in metabolic studies *in vivo* and *in vitro* (Figs. 1 and 2).

The most frequently studied metabolic reactions of testosterone are oxidoreductive reactions at positions 3, 4, and 17, and C-hydroxylations, the latter reactions being catalysed exclusively by cytochrome P450 families of enzymes (1-3). Other reactions include aromatisation of the A ring (4), formation of Δ^6-T and Δ^6-T-6,7-oxide (5,6), and as an interesting example 19-nortestosterone was indentified as metabolite in mouse kidney (1).

Intention of this presentation is to bring out selected examples of the metabolic reactions and formation of the end-products of testosterone metabolism. These considerations could be of interest in determination of testosterone metabolites in urine samples, to enable better understanding and interpretation of the analytical results.

Different approaches and experimental models are used when studying metabolism of testosterone and other steroids. Studies are performed *in vivo* and *in vitro*, using different animal models and with humans in clinical studies. *In vitro* experiments are performed using variety of steroidogenic and non-steroidogenic tissues, cell cultures and cell fractions, including purified enzymes and reconstituted enzyme systems. The latter experimantal models are used particulary to investigate specificity and mechanisms of metabolic reactions (Fig. 3).

The main characteristics of metabolic reactions of testosterone are presented in Fig. 4. These characteristics can be extended also to metabolism of other endogenous and synthetic steroids as well. Particulary intersting features are exercised by cytochrome P450 (cyt. P450) catalysed reactions. Testosterone is oxygenated by cyt. P450 isoenzymes with high degree of stereo- and regiospecificity and is repeatedly used as a highly specific substrate

for these enzymes. Testosterone as a model substrate is convenient when studying the level of particular isoenzyme in the test system, as well as when studying other factors and mechanisms of steroid metabolic reactions (3).

Another important feature is that steroid hormones, including testosterone, play an important role in regulating the expression of several constitutive hepatic and other tissues cyt. P450s (3).

Oxidoreductive reactions
Interconversion of testosterone and epitestosterone

Interconversion between testosterone and its 17α-isomer epitestosterone *in vivo* was observed following intravenous infusion of high doses either of the isomer. Administration of the single steroid resulted in increased urinary excretion both the testosterone and the epitestosterone glucuronides (Fig. 5) (10). In the same study administration of androstenedione also led to increased excretion of both glucuronides. The interconversion was also observed in *in vitro* studies (Fig. 6). For example, epitestosterone was identified when testosterone was incubated with human peripheral venous blood (11), and with microsomal preparations from kidneys of rabbits, but not with the same from human or male rat (12). In the latter experiments performed by Takeyama *et al.* (13) showed conversion of ^{14}C-androstenedione and H^3-progesterone to both of the isomers (Fig. 6). The conversion occured in neonatally grafted mouse testes in female, but not in male or normal mouse testes.

Formation of androstenedione from testosterone and epitestosterone was reported by Wood *et al.* (14). From the differences in enrichement with ^{18}O in androstenedione formed from testosterone and epitestosterone as a substrates (5-8% and 84%, respectively), different mechanisms of androstenedione formation from the isomers were proposed. Depending on the substrate used (testosterone, androstenedione, or epitestosterone) difference in the ratios of C-16 hydroxylated α- and ß-isomers were found as well (Fig. 7). The enzyme used was cyt. P450 purified from hepatic microsomes of Aroclor treated male rats.

Several enzymes (Fig. 5) can catalyse oxidoreductive reaction in C-17: 17α- and 17ß-hydroxysteroid dehydrogenases, and different isoforms of cyt. P450 as well (Fig. 14, ref. 7).

Formation of diols, triols and 5α-reduction

Diols of testosterone (3α- and 3ß-androstanediols) are formed by reduction of C3-carbonyl during the reaction catalysed by 3α- and 3ß-hydroxysteroidoxidoreductase (HSOR), respectively, following previous reduction to 5α-dihydrotestosterone (5α-DHT). Following administration of ^{13}C-testosterone (<0.2% of the dose) and 3α-androstanediol (<2% of the dose) were identified. ^{13}C-5 α-DHT was identified only in plasma samples. While 3α-diol was identified as an end-product of testosterone metabolism. 3ß-diol undergoes further metabolism (Fig. 8, ref. 15,16,19). It was concluded that determination of 3α-diol in urine sample can be used as a measure for conversion of testosterone to biologocally important 5α-DHT. In *in vitro* studies (17,18) both the 3α- and 3ß-isomers are formed from testosterone, however, the concentration of 3ß-diol in human testicular and prostate tissue was higher than that of 3α-isomer. The 3ß-isomer is further metabolised to 3ß-, 7α-triol, 3ß-, 7α-, 17-one and 3ß-, 6α-,17-one (Fig. 9). It was concluded that the major metabolite of 5α-DHT is 3ß-diol.

Following *in vivo* administration of ^{14}C 3ß-diol, 3α- and 3ß-triols as well as 3α-diol and androsterone were identified as major metabolites in urine samples. Urinary excretion of 3α-diol and androsterone was 1% and 20% of the dose, respectively (Fig. 9, ref. 19). Formation both of diol isomers from 5α-DHT was also shown by incubating the latter compound *in vitro* with prostate tissue preparations (20).

Testosterone can be reduced to 5α-DHT either directly or after previous hydroxylation at position 6ß- (Fig. 1). The 6α- and 7α-isomers, however, are blocking the enzyme catalysing the 5α-reduction reaction. Good substrates for steroid 5α-reductase are androstenedione and testosterone metabolites hydroxylated at the positions 14α-, 16α-, and 16ß- of the 5 membered ring (21).

Hydroxylations of testosterone

Hydroxylations of testosterone are catalysed by isomers of cytochrome P450, in which reactions they act with a high degree of regio- and stereoselectivity (3). Hydroxylated metabolites are the major metabolites formed from testosterone by livers of different animals. In stereoidogenic tissues cytochrome P450 catalyse reactions which constitute important steps in biosynthetic pathways of steroids (1,2).

Testosterone can be oxygenated to 21 monohydroxy isomers, theoretically (22). Rat liver microsomes, for example, oxygenate testosterone to 10 different compounds (Fig. 10, ref.

21). The profile of testosterone metabolites excreted in urine, as well as those formed in *in vitro* studies, can be influenced and regulated by treatment of animals with drugs and chemicals known to behave as inducers (22-26) or inhibitors (27) of cytochrome P450 enzymes. Examples of drugs reported to change the composition of cytochrome P450 isoenzymes, and therefore metabolic profile of testosterone, in liver cells are shown in Fig. 12. Pretreatment of animals with, for example, phenobarbital, 3-methylcholanthrene (3-MC), or PCN (pregnenolone-16α-carbonitrile) increase cytochrome $P450_b$, $P450_c$, and $P450_p$ up to 100 fold (24).

Miura *et al.* (28) reported qualitative as well as quantitative differences between species (comparison was made for male and female mice, rabbits, guinea pigs, hamsters, dogs and humans) in hydroxylation of testosterone at position 2α-, 2ß-, 7α-, 15α-, 16ß-, C-19, and 17-oxidation. Difference in 15α-hydroxilation between mice and human microsomal tissue preparations was also reported by Mäenpää *et al.* (29).

Wood *et al.* (30) investigated *in vitro* metabolism of testosterone and androstenedione using five highly purified cytochrome P450 isoenzymes. The positional specificity and stereoselectivity of the isoenzymes tested was also dependent on the steroid used as a substrate. Regio- and stereoselective activity of isoenzymes of cyt. P450 is indicated in Fig. 13. Based on the results obtained in *in vitro* studies (29,31) hydroxylated metabolites most likely to be formed in human liver are 2ß-, 6ß-, 15ß-OH (Fig. 14). Human liver also contained some 2α- and low 15α-OH activity (29).

In summary, metabolites which would be expected as end-products after testosterone administration are 3ß- and 3α-, 17ß-diols (A), and hydroxylated testosterone (B) at different positions shown in Fig. 15.

It can be well assumed that under the conditions of long- or short-term misuse or natural (testosterone) and/or synthetic steroids, the endogenous or "natural" profile of steroid-inducible cyt. P450 isoenzymes in liver (and possibly in other tissues as well) will be modulated. As a consequence of such a modulation, capacity of hepatic tissue to form one or more metabolic end-products of testosterone (Fig. 15) will be modified and will result in changed profile of the products excreted as well. Formation of the end-products can be influenced by: induction of hydroxylation pathways (for example, 6ß-hydroxylation of tetosterone catalysed by IIIA1 isoenzyme, is induced by exposure to synthetic steroids, ref. 3); hormonally regulated pathways can be suppressed or induced by administration of steroids; catalytic activity of cytochrome P450 isoenzymes can be inhibited by administered steroids (stanozolol was reported to inhibit cyt. P450 catalysed reactions, ref. 32). Quantitative determinatin of the end-products of testosterone metabolism, or determination

of the products ratios might be useful as an additional criterion when confirming medically unjustified administration of natural and synthetic steroids.

Literature

1. C.D. Kochakian, Steroids 55, 92 - 97, 1990.
2. P.E. Hall, Clin. Exp.Pharmacol. Physiol. 16, 485 - 489, 1989.
3. D.J. Waxman, Biochem. Pharmacol. 37, 71 - 84, 1988.
4. M. Finkelstein *et al.* Endocrinology 108, 943 - 947, 1981.
5. K. Korzekwa *et al.*, Drug. Metabol. Rev. 20, 535 - 539, 1989.
6. J.R. Gillette, Life Sciences 49, 1 - 14, 1991.
7. B. Bartlomowicz *et al.*, Biochem.Biophys.Res.Commun. 160, 46 - 52, 1989.
8. M.J. Namkung *et al.*, Mol. Pharmacol. 34, 628 - 637, 1988.
9. H.M. Wortelboer *et al.*, Biochem. Pharmacol. 40, 2525 - 2534, 1990.
10. J. Tamm *et al.*, Steroids 6, 659 - 669, 1969.
11. J. Blaquier *et al.*, Acta Endocrinol. 54, 208 - 214, 1967.
12. P. Uotila *et al.*, J. Biochem. 12, 345 - 351, 1980.
13. M. Takeyama *et al.*, J.steroid Biochem. 25, 121 - 126, 1986.
14. A.W. Wood *et al.*, J. Biol.Chem. 263, 17322 - 17332, 1988.
15. H. Vierhapper *et al.*, J.steroid Biochem. 29, 105 - 109, 1988.
16. H. Vierhapper, Steroids 55, 177 - 180, 1990.
17. J. Tamm *et al.*, J.steroid Biochem. 26, 345 - 348, 1987.
18. P. Ofner *et al.*, J.steroid Biochem. 18, 415 - 423, 1983.
19. F. Jacolot *et al.*, J.steroid Biochem. 14, 663 - 669, 1981.
20. Kang - Hyun Lee and P. Ofner, J.steroid Biochem. 29, 553 - 557, 1988.
21. A.J. Sonderfan and A. Parkinson, Arch.Biochem. Biophys. 256, 208 - 218, 1988.
22. D.W. Lee and K.H. Park, Int. J. Biochem. 21, 49 - 57, 1989.
23. C. Vind *et al.*, Arch Biochem.Biophys. 275, 140 - 150, 1989.
24. A.J. Sonderfan *et al.*, Arch.Biochem.Biophys. 255, 27 - 41, 1987.
25. M. Kelley *et al.*, Biochem.Pharmacol. 39, 1991 - 1998, 1990.
26. K. Morita *et al.*, J. Pharmacobio-Dyn. 11, 808 - 815, 1988.
27. R.A. Galbraith *et al.*, Biochem. Pharmacol. 38, 2046 - 2049, 1989.
28. T. Miura *et al.*, Japan. J. Pharmacol. 49, 365 - 374, 1989.
29. J. Mäenpää *et al.*, Biochem.Pharmacol. 42, 1229 - 1235, 1991.
30. A. Wood *et al.*, J.Biol.Chem. 258, 8839 - 8847, 1983.
31. D.J. Waxman *et al.*, Arch.Biochem. Biophys. 263, 424 - 436, 1988.
32. S. Rendic and H.H. Ruf, Biochem.Pharmacol. 37, 768 - 770, 1989.

METABOLISM OF TESTOSTERONE

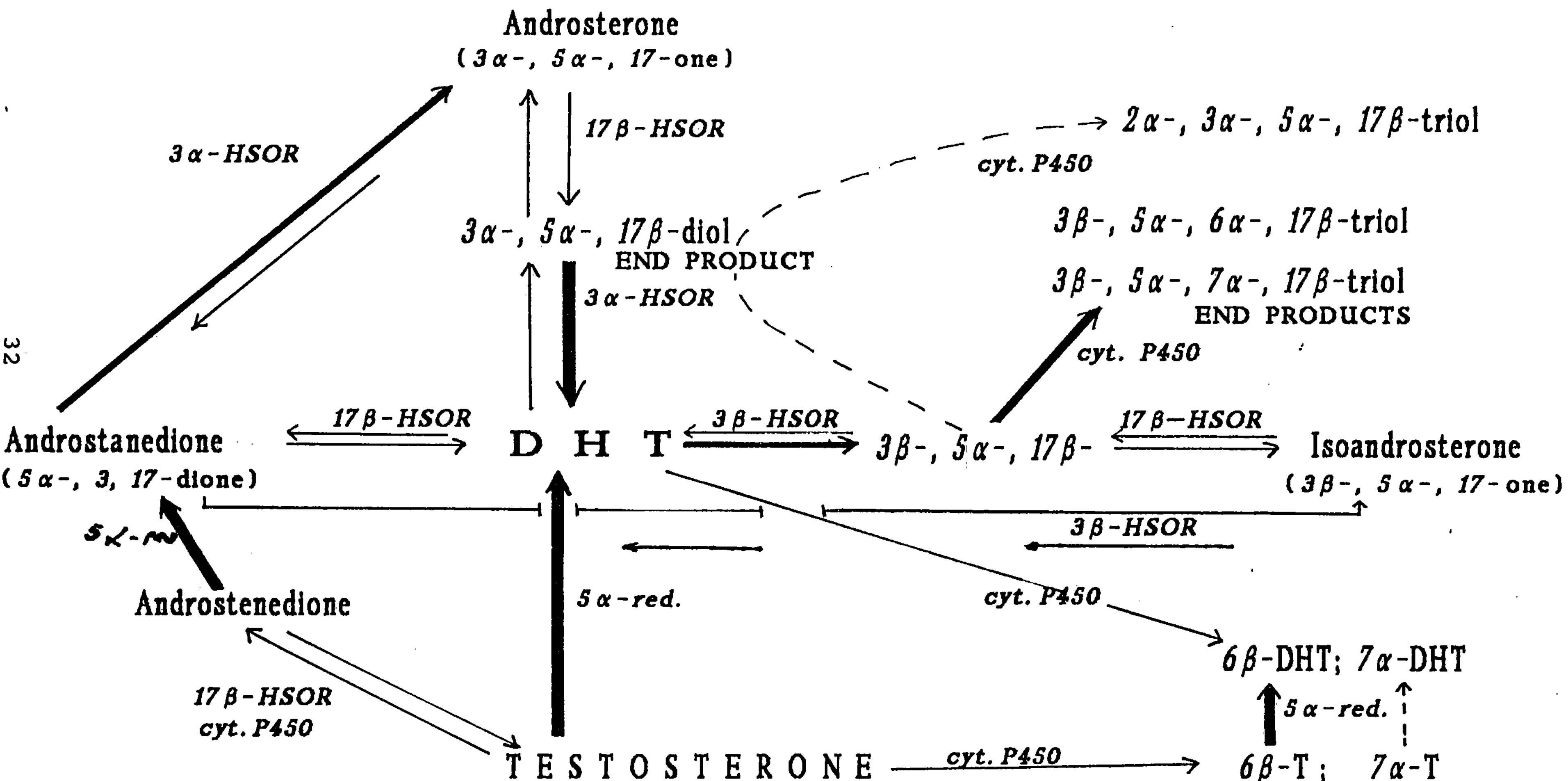


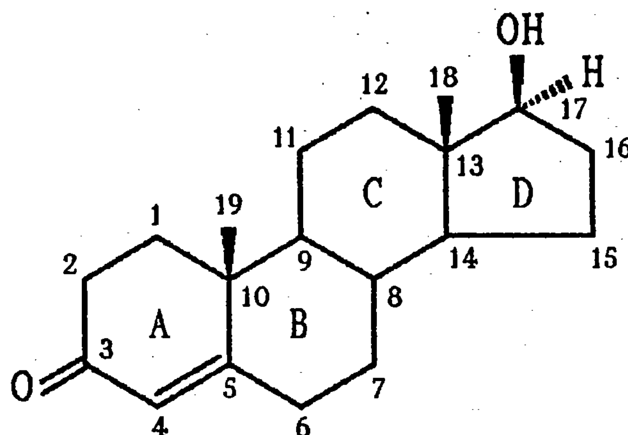
Fig. 1

TESTOSTERONE METABOLISM

TESTOSTERONE ⟶ 27 other steroids

+ 548 different monohydroxy derivatives
(in 9 positions)

+ ? no. of polyhydroxylated derivatives



REACTIONS	ENZYMES
5α-, and *5β*-reductions	*5α*-, and *5β*-reductase
3α-, and *3β*-oxidations/red.	*3α*-, and *3β*-hydroxysteroid oxidoreductase (HSOR)
C-hydroxylations (α-, and β-)	isoforms of cyt.P450
17β-oxidation	*17β*-hydroxysteroid oxidoreductase (HSOR)

- - - - - - - - - - - - - - - - -

aromatisation

formation of :

- 6-keto group
- 19-nortestosterone
- 4,6-diene (Δ^6 T)

Fig. 2

APPROACHES TO METABOLIC STUDIES

IN VIVO

Clinical studies (humans):
- healthy
- in disease

Animal studies:
- untreated
- pretreated

IN VITRO
(human or/and animal)

- isolated organs
- cell cultures
- cell fractions
- purified enzymes (pretreatment?)

Fig. 3

CHARACTERISTICS OF METABOLIC REACTIONS
- TESTOSTERONE -

1. strict regio- and stereospecificity of different isoenzymes
2. sex specificity
3. inducibility of isoforms by drugs, chemicals, including steroids
4. inhibition by drugs and chemicals
5. species and interindividual variations
6. age dependence
7. hormonal status

Fig. 4

INTERCONVERSION OF TESTOSTERONE ⇄ EPITESTOSTERONE

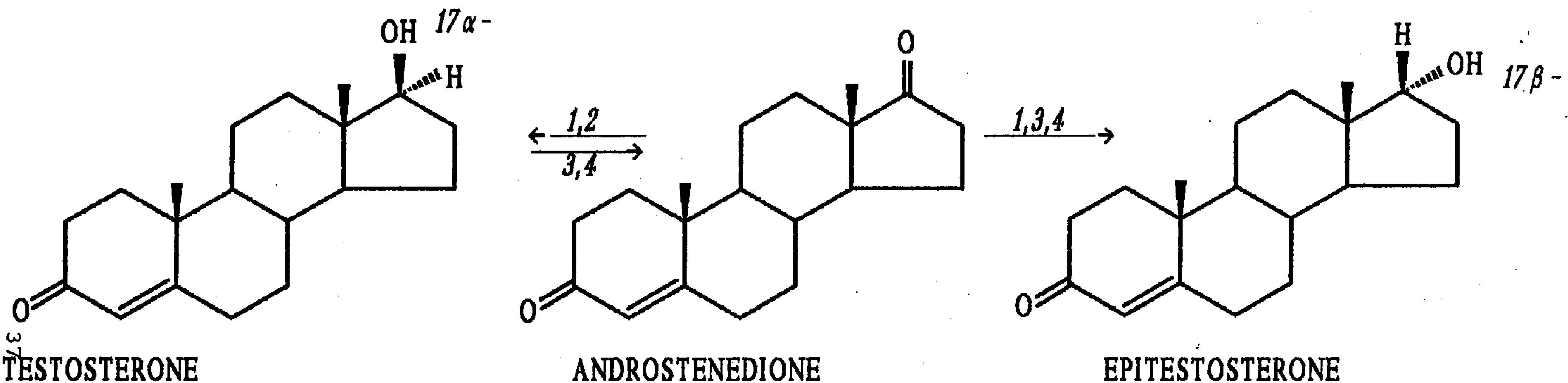
OBSERVED: After high dosis of *T* or *ET* (following *i.v. infusion*, *oral* or *i.m.*) in humans (*Tamm et al.* 1966)

ENZYMES:

- *17α*- and *17β*-oxidoreductase
- hepatic microsomal cyt.P450
- hepatic mitochondrial cyt.P450 (treatment with *T* reduced enzyme level)

Fig. 5

ANDROSTENEDIONE AND TESTOSTERONE CONVERSION
(*in vivo* and *in vitro* studies)



<u>MODEL</u>:

1. grafted mouse testes in female mice (*Takeyama et al.*, 1986)

2. normal mouse testes (*Takeyama et al.*, 1986)

3. *in vitro* kidneys (from hamsters, rabbit, mause)
   - not observed in human and rat

   (*Uotila et al.*, 1981)

4. *in vitro*, in venous blood and adrenal tissue from man and women
   (*Blaquier et al.*, 1967)

Fig. 6

PURIFIED ENZYMES:

Rat hepatic, Aroclor pretreated

Wood et al.,1988.

Fig. 7

ENZYMES:

Cytochrome P450 (II B1 rat)

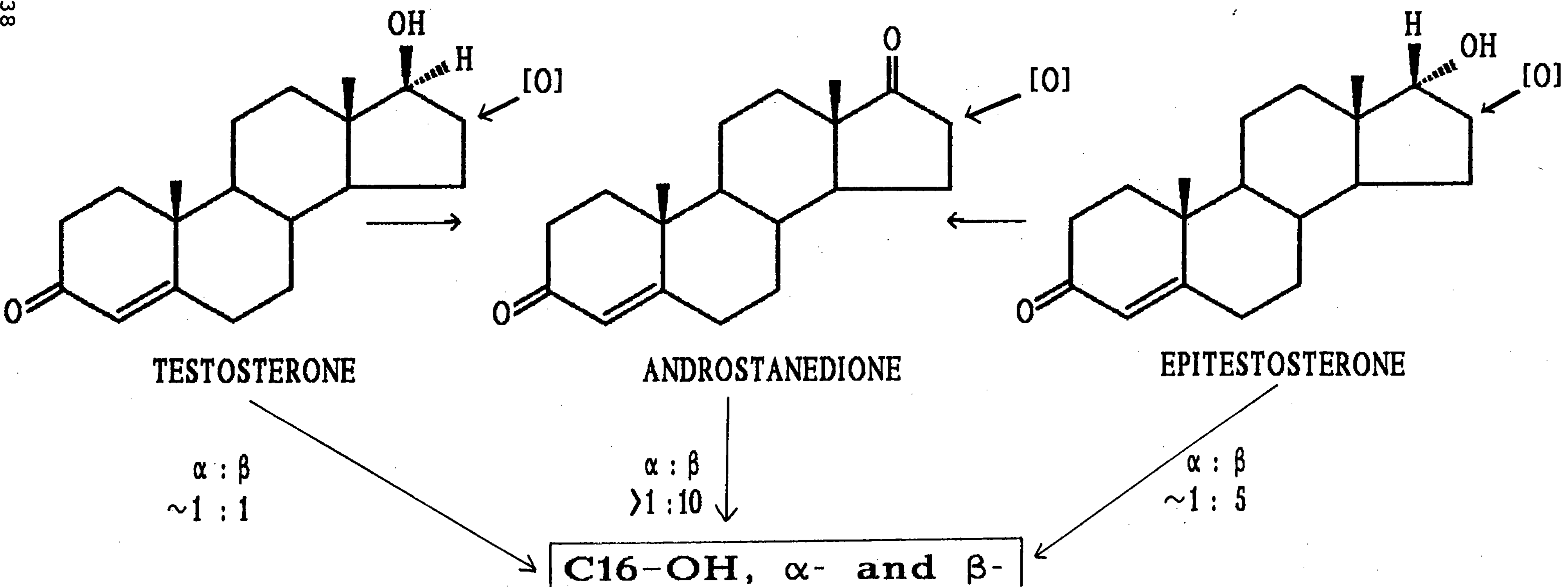
Cytochrome P450 reductase

NADPH, $^{18}O_2$

OTHER ENZYMES:

17β-OH hydroxysteroid dehydrogenase

and other cyt. P450



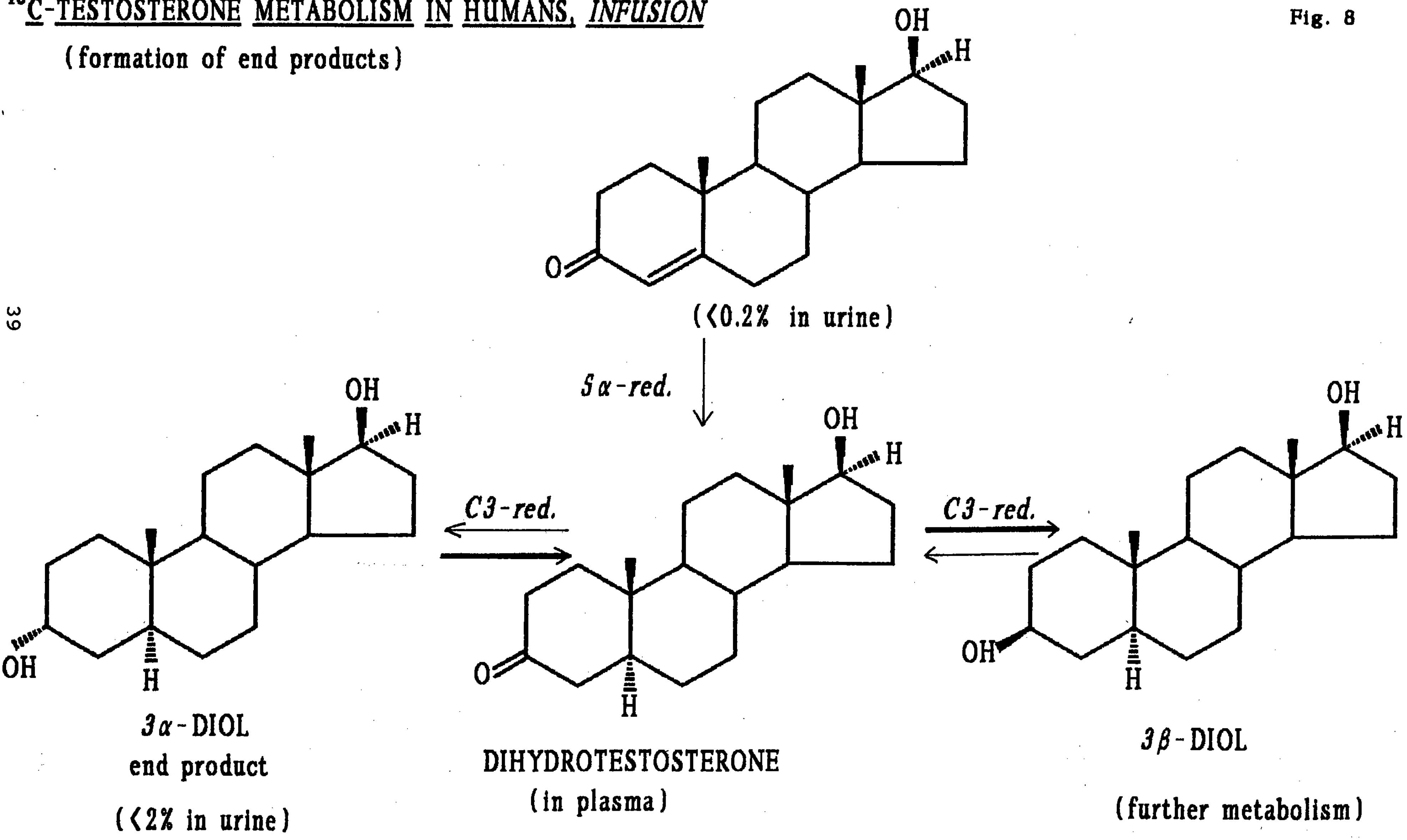
FORMATION OF DIOLS

Vierhapper, 1988.
1990.

Fig. 8

^{13}C-TESTOSTERONE METABOLISM IN HUMANS, *INFUSION*

(formation of end products)



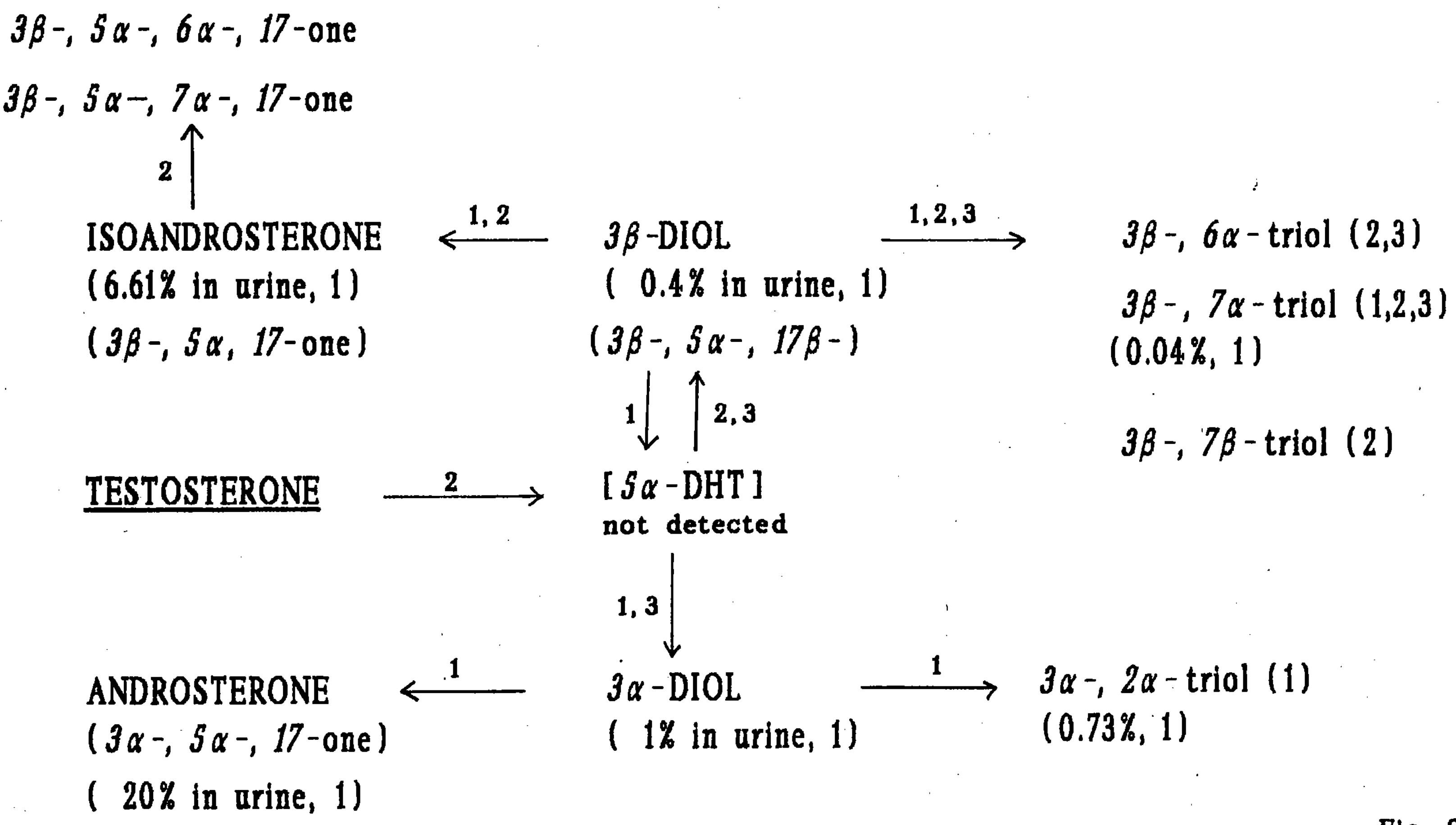


Fig. 9

C-HYDROXYLATIONS OF TESTOSTERONE

ENZYMES: Cytochrome P450 isoforms

MAIN CHARACTERISTICS: regioselectivity
stereoselectivity
species, sex and organ dependence
hormonal regulation

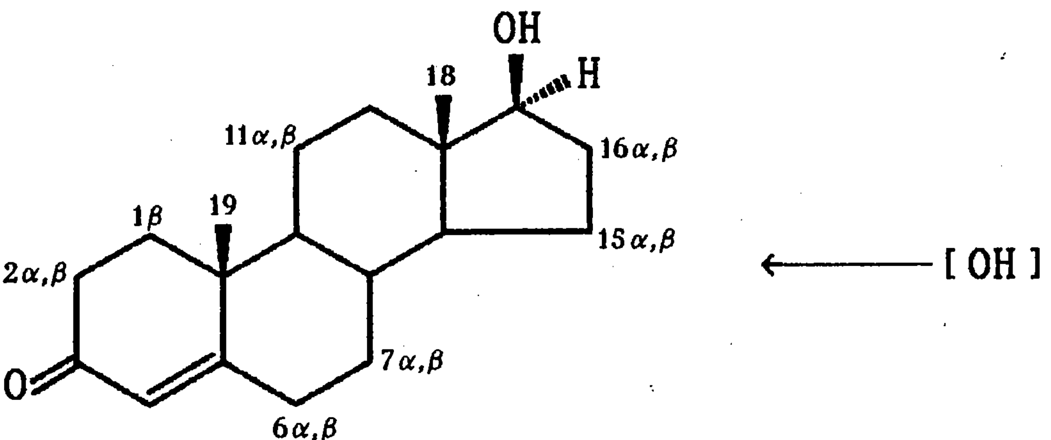


Fig. 10

SPECIES DEPENDENCE (*IN VITRO*):

Source of microsoms	Position of hydroxylation
Human liver (M)	2β-, 6β-
Rat liver (M)	2α-, 6β-, 16α-, 17-oxid.
Rat liver (F)	6β-, 7α-
Mice liver (F, M)	6β-, 7α- 16α-
Rabbit	6β-, 16α-
Hamster	2β-, 6β-, 7α-, 15α-, 19-
Dogs	2β-, 6β-, 16α-

SEX DEPENDENCE (RAT, *IN VITRO*)

	F / M
6β-OH	1 : 10
7α-OH	3 : 1
17-OH oxid.	1 : 6

Miura et al., 1989

Fig. 11

ISOFORM SPECIFICITY *IN VITRO*:
(from rat liver microsomes)

Isoform:	Position of hydoxylation:									
cyt. $P450_p$[1]		2β-,		6β-,		15β-,				18-
$P450_a$[2]			6α-,		7α-					
$P450_b$[3]							16α-,	16β-,	17-ox	
$P450_h$[4]	2α-,			6β-,			16α-,		17-ox.	
$P450_c$[5]		2β-,		6β-						

Pretreatment:	
Aroclor	2,3,5
Phenobarbital	1,2,3
Erythromycin	1
3-MC	2,5
PCN	1
Rifampicin	1
Dexamethasone	1
Spironolactone	1
Troleandomycin	1

Vind et al., 1989.
Sonderfan et al., 1987.
Kelley et al., 1990.

Fig. 12

ISOFORMS OF CYT. P450 WHICH HYDROXYLATE TESTOSTERONE

Isoform		Position									
		2α–	2β-	6α-	6β-	7α-	15β–	16α–	16β-	17β-oxid.	18 C-
I	A1			+		+					
	A2	+			+			+			
II	A1					+					
	B1							+	+	+	
	B2							+	+	+	
	C11	+						+		+	
	C12						+				
III	A1		+		+		+		+		+
	A2				+						
	A3							+			
	A4				+						

Wortelbour et al., 1990.
Namkung et al., 1988.
Bartlomowitz et al., 1989.

Fig. 13

HUMAN LIVER MICROSOMES - MAJOR ROUTES OF OXIDATIVE INACTIVATION

6β-OH T > 75 % of total hydroxylated metabolites

and

2β-OH T ~10% of 6β-

15β-OH T 3-4% of 6β-

Waxman, 1988.

Fig. 14

S U M M A R Y :

<u>METABOLITES OF *T* IN STEROIDOGENIC AND OTHER TISSUES, AND EXCRETED IN HUMAN URINE</u>

A) 3β-, 5α-, 17β-DIOLS

3α-, 5α-, 17β-DIOLS

B) 2β-OH, 2α-OH

6α-OH, 6β-OH

7α-OH, 7β-OH

15β-OH, 15α-OH

16β-OH

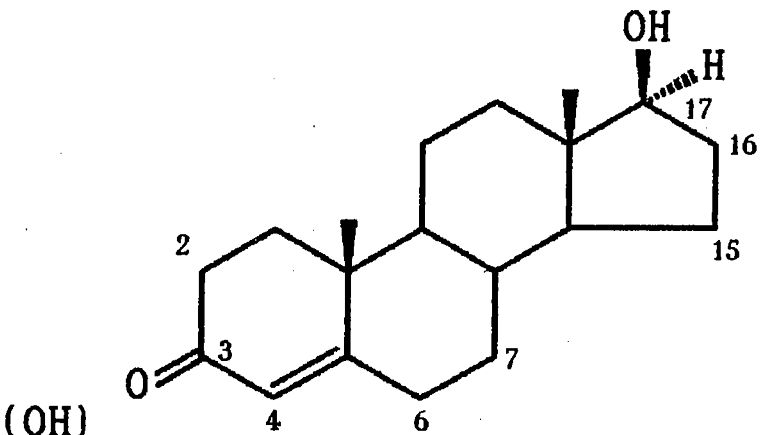


Fig. 15