ON TESTICULAR STEROID BIOSYNTHESIS

INHIBITION OF $C_{17,20}$-LYASE ACTIVITY

THE ROLE OF Δ^5,3β-HYDROXYSTEROIDS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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aan Tineke, Edwin en Jasper.

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Contents

List of trivial names*

androstenediol	- 5-androstene-3β,17β-diol
androstenediol sulfate	- 17β-hydroxy-5-androsten-3β-yl sulfate
androstenetrione	- 4-androstene-3,6,17-trione
cyanoketone	- 2α-cyano-4,4',17α-trimethyl-17β-hydroxy-5-androsten-3-one
dehydroepiandrosterone	- 3β-hydroxy-5-androsten-17-one
dehydroepiandrosterone sulfate	- 17-oxo-5-androsten-3β-yl sulfate
11-deoxycortisol	- 17α,21-dihydroxy-4-pregnene-3,20-dione
20α-dihydroprogesterone	- 20α-hydroxy-4-pregnen-3-one
20β-dihydroprogesterone	- 20β-hydroxy-4-pregnen-3-one
20β-dihydroprogesterone chloroacetate	- 3-oxo-4-pregnen-20β-yl monochloro-acetate
7α-hydroxyandrostenedione	- 7α-hydroxy-4-androstene-3,17-dione
17α-hydroxyprogesterone	- 17α-hydroxy-4-pregnene-3,20-dione
17α-hydroxypregnenolone	- 3β,17α-dihydroxy-5-pregnen-20-one
7α-hydroxytestosterone	- 7α,17β-dihydroxy-4-androsten-3-one
isoxazole	- 17β-hydroxy-4,4',17α-trimethyl-5-androsten-[2,3d]-isoxazole
melatonin	- 5-methoxy-n-acetylserotonin
pregnenetrione	- 4-pregnene-3,6,20-trione
serotonin	- 5-hydroxytryptamine
testosterone chloroacetate	- 3-oxo-4-androsten-17β-yl mono-chloroacetate

*Other trivial names for steroids are given according to the IUPAC-IUB 1967 Revised Tentative Rules for Steroid Nomenclature

List of enzymes

trivial name	systematic name	number
3α-hydroxysteroid dehydrogenase	3α-hydroxysteroid:NAD(P) oxidoreductase	E.C. 1.1.1.50
3β-hydroxysteroid dehydrogenase	3β-hydroxysteroid:NAD(P) oxidoreductase	E.C. 1.1.1.51
17β-hydroxysteroid dehydrogenase	17β-hydroxysteroid:NAD(P) oxidoreductase	E.C. 1.1.1.64
5α-reductase	5α-steroid:NAD(P) Δ^4-oxidoreductase	E.C. 1.3.1.99
17α-hydroxylase	progesterone-NADPH:oxygen oxidoreductase (17α-hydroxylating)	E.C. 1.14.1.7
21-hydroxylase	steroid-NADPH:oxygen oxidoreductase (21-hydroxylating)	E.C. 1.14.1.18
steroid sulfokinase	3'-phosphoadenylylsulfate:3β-hydroxysteroid sulfotransferase	E.C. 2.8.2.2
esterase	aspecific esterase	E.C. 3.1.1.1
steroid sulfatase	3β-hydroxysteroid-sulfate sulfo-hydrolase	E.C. 3.1.6.2
$C_{17,20}$-lyase	17α-hydroxy,20-oxosteroid:$C_{17,20}$,acetate lyase	

List of abbreviations

ACTH	- adrenocorticotropic hormone
3',5'-cAMP	- 3',5'-adenosine monophosphate (cyclic)
Ci	- curie
dpm	- desintegrations per minute
EDTA	- ethylenediamine tetraacetate
g.l.c.	- gas-liquid chromatography
h	- hour
HCG	- human chorionic gonadotropin
K_i	- inhibitor constant
K_m	- Michaelis constant
LH	- luteinizing hormone
min	- minute
NAD^+	- nicotinamide-adenine dinucleotid (oxidized)
NADPH	- nicotinamide-adenine dinucleotid phosphate (reduced)
S.E.M.	- standard error of the mean
S.D.	- standard deviation
t.l.c.	- thin layer chromatography
Tris	- 2-amino-2-hydroxymethylpropane-1,3-diol

General introduction

Pathways and control of steroid biosynthesis

The hormones secreted by the testis, the androgens, maintain and control the development of the male accessory reproductive organs and secondary sexual characteristics. There are two principal androgens, testosterone and androstenedione and they are synthesized in the interstitial tissue of the testis.

It is generally accepted that these steroids are derived from cholesterol and are formed along the Δ^5,3β-hydroxysteroid pathway and the Δ^4,3-oxosteroid pathway[1,2,3] (fig. 1). The first steps in steroidogenesis are basically the same in the mammalian testis, ovary and adrenal gland. Androstenedione and testosterone are precursors for ovarian oestrogen biosynthesis, while progesterone and 17α-hydroxyprogesterone are utilized in the formation of corticosteroids in the adrenal cortex.

It has been shown that in the hog adrenal gland the cholesterol side-chain cleaving enzyme is localized in the inner membrane of the mitochondria[4]. A major portion of the pregnenolone is probably formed by an enzyme-mediated attack of oxygen on cholesterol, resulting in the formation of 20α,22R-dihydroxycholesterol, which is subsequently oxidatively cleaved to pregnenolone[5]. Pregnenolone leaves the mitochondria and is converted to testosterone by enzymes, localized on the smooth-surfaced endoplasmatic reticulum[6]. Considerable information is available on the properties of the Δ^5,3β-hydroxysteroid dehydrogenase and its mechanism of action; it is a single, NAD^+-dependent enzyme oxidizing all four Δ^5,3β-hydroxysteroids[1,7]. In contrast, the presence of several substrate-specific Δ^5,Δ^4-isomerases has been demonstrated in bovine adrenal gland preparations[8,9]. The cholesterol side-chain cleaving enzyme and the $C_{17,20}$-lyase show the same requirements as the steroid hydroxylation reactions i.e. NADPH, oxygen and the presence of cytochrome

P_{450}[6,10]. The 17α-dehydrogenation is reversible and NAD(H)/NAD(P)H-dependent. The presence of steroid sulphokinase and steroid sulphatase activities and of enzymes which metabolize steroid hormones is described later in this thesis. In the rat testis the Δ^4,3-oxosteroid pathway is the preferred route for testosterone biosynthesis, while both pathways are involved in the testes of man, dog and rabbit[1,2,3].

Testosterone production in the testis is regulated by an interplay with the hypophysial hormone LH. Administration of LH (or HCG) leads to an increased testosterone production through the stimulation of the conversion of cholesterol to pregnenolone which is the rate-limiting step in steroidogenesis. Androgens in turn regulate LH secretion through the negative feedback effect of testosterone on the hypothalamus and hypophysis. Decreased levels of circulating androgens result in an increased LH secretion by the hypophysis[11]. The biochemical mechanism through which LH exerts

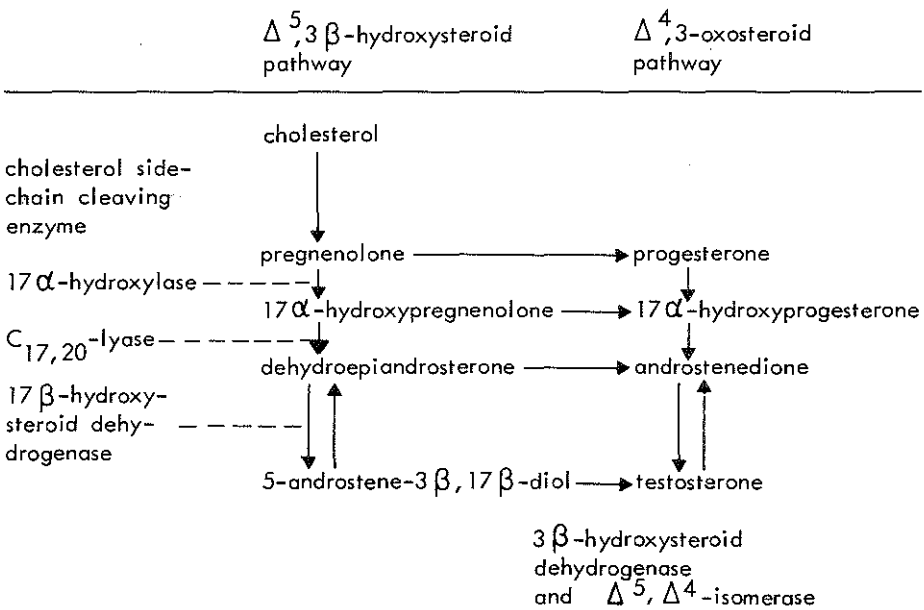


Fig. 1 Pathways and enzymes involved in testosterone biosynthesis

its effect is largely unknown. In interstitial tissue of rat testis LH stimulates the formation of 3',5'-cAMP and testosterone and the increase in 3',5'-cAMP production occurs before the increase in testosterone production[12,13]. A long term effect of LH in testis is to increase the activities of Δ^5,3β-hydroxysteroid dehydrogenase, 17α-hydroxylase and $C_{17,20}$-lyase[1,2].

Another possible control mechanism in regulating testosterone production is inhibition by steroids at the cellular level. It is known that several steroids can inhibit the in vitro activity of enzymes required for steroid biosynthesis. In rat testis the $C_{17,20}$-lyase can be inhibited by 17α,20α-dihydroxy-4-pregnen-3-one, a metabolite of 17α-hydroxyprogesterone[14].

The first part of this thesis describes an investigation in which the role of 17α,20α-dihydroxy-4-pregnen-3-one in the in vivo testosterone production is evaluated.

The second part of this thesis deals with the role of Δ^5,3β-hydroxysteroids as precursors and the relative importance of alternative pathways. The significance of the Δ^5,3β-hydroxysteroid pathway and androstenediol in testosterone biosynthesis in rabbit testis was measured. In addition, the existence of precursors of dehydroepiandrosterone and androstenediol other than 17α-hydroxypregnenolone was investigated.

References

1 Hall,P.F.(1970)in "The Testis"(Johnson,A.D., Gomes,W.R. and VanDemark,N.L.,eds),vol.2, pp.1-71,Academic Press,New York

2 Eik-Nes,K.B.(1970)in "The Androgens of the Testis" (Eik-Nes,K.B.,ed),pp.1-47,Marcel Dekker,New York

3 Hall,P.F.(1970)in "The Androgens of the Testis"(Eik-Nes,K.B., ed),pp.73-115,Marcel Dekker,New York

4 Yago,N., Kobayashi,S., Sekiyama,S., Kurokawa,H., Iway,Y., Suzuki,I. and Ichii,S.(1970)J.Biochem. 68,775-783

5 Burnstein,S., Kimball,H.L. and Gut,H.(1970)Steroids 15,809-857
6 Inano,H., Inano,A. and Tamaoki,B.(1970)J.Steroid Biochem. 1, 83-91
7 Murota,S., Fenselau,C.C. and Talalay,P.(1971)Steroids 17,25-37
8 Ewald,W., Werbin,H. and Chaikoff,P.L.(1964)Steroids 4,759
9 Ewald,W., Werbin,H. and Chaikoff,P.L.(1965)Biochim.Biophys.Acta 111,306
10 Machino,A., Inano,H. and Tamaoki,B.(1969)J.Steroid Biochem. 1, 9-16
11 Schwartz,N.B. and McCormack,C.E.(1972)Annu.Rev.Physiol. 34, 425-471
12 Rommerts,F.F.G., Cooke,B.A., van der Kemp,J.W.C.M. and van der Molen,H.J.(1972)FEBS(Fed.Eur.Biochem.Soc.)Lett. 24, 251-254
13 Cooke,B.A., van Beurden,W.M.O., Rommerts,F.F.G. and van der Molen,H.J.(1972)FEBS(Fed.Eur.Biochem.Soc.)Lett. 25, 83-86
14 Inano,H., Nakano,H., Shikita,M. and Tamaoki,B.(1967)Biochim. Biophys.Acta 137,540-548

Part 1 Inhibition of $C_{17,20}$-lyase activity

1.1 Inhibition of steroid hormone biosynthesis

1.1.1 Introduction

Under in vitro conditions a large number of steroids can inhibit the enzymes involved in the biosynthesis of steroid hormones. Such steroid inhibitors can be found for almost every step in steroid hormone biosynthesis, possibly as a consequence of the basic structural properties which steroids have in common. Some of these inhibitors are naturally occurring steroids and therefore the presence of a control of steroid hormone biosynthesis at the cellular level is often postulated.

The occurrence of inhibition of steroid biosynthesis by steroids under in vivo conditions is difficult to establish. The description of inhibition has been restricted thusfar to observations of decreased conversions in vitro. Enzyme kinetics and the characteristics of inhibition have been studied in only a few cases and this has resulted in information on K_m- and K_i-values, which is necessary for understanding the significance of inhibition as a regulating factor. Constants were determined for the inhibition of $C_{17,20}$-lyase by 17α,20α-dihydroxy-4-pregnen-3-one[1,2] and for the inhibition of Δ^5,3β-hydroxysteroid dehydrogenase by 7α-hydroxy-androstenedione[3] as well as the inhibition of steroid sulfatase by various steroids[4-8].

The best impression about the physiological significance of such inhibitors may be derived from enzyme kinetic studies combined with information on the availability of a potential inhibitor at the site of steroid biosynthesis. On the basis of such data, Wiener and Allen[9,10] have proposed that inhibition of the 20α-hydroxysteroid dehydrogenase by oestrogens is part of the control mechanism in placental steroid biosynthesis of 20α-dihydroprogesterone. Jensen

et al.[11,12] used similar arguments when discussing the effect of dehydroepiandrosterone on the $C_{17,20}$-lyase activity in the human adrenal gland.

The lack of information on endogenous tissue levels and the cellular localization of potential inhibitors hamper the evaluation of the inhibitor for the in vivo situation. Some authors have described effects of steroids which are present in relatively low or unknown concentrations, or which are not present at all at the site of steroid biosynthesis. This includes the inhibitory effects reported for 20α(and β)-dihydroprogesterone[1,13], oestradiol[14] and deoxycorticosterone in the rat testis, and 11-deoxycortisol and cortisol in the rat adrenal gland[15-18]. Some of the investigated inhibitors are, however, formed at their presumed cellular site of action. For instance, the effect of corticosterone on mitochondrial NADH-oxidase may very well be of importance for the rate of 11β-hydroxylation in the rat adrenal, because low concentrations (10 μM) are required for inhibition[19] and because the concentration in adrenal mitochondria is probably higher than at any other (sub)cellular site in the rat.

A detailed description of inhibition of steroid biosynthesis by steroids is given in paragraph 1.1.3.

1.1.2 Scope of this investigation

Most of the studies on the inhibition of steroid biosynthesis by steroids have been carried out in vitro. There have been few attempts to test the significance of in vitro results under in vivo conditions. It was decided therefore to investigate one potentially important inhibitor of steroid biosynthesis, 17α,20α-dihydroxy-4-pregnen-3-one and its effect on testis $C_{17,20}$-lyase under in vivo conditions.

Inano and co-workers[1,2] have suggested that 20α-hydroxysteroid dehydrogenase might regulate testosterone biosyn-

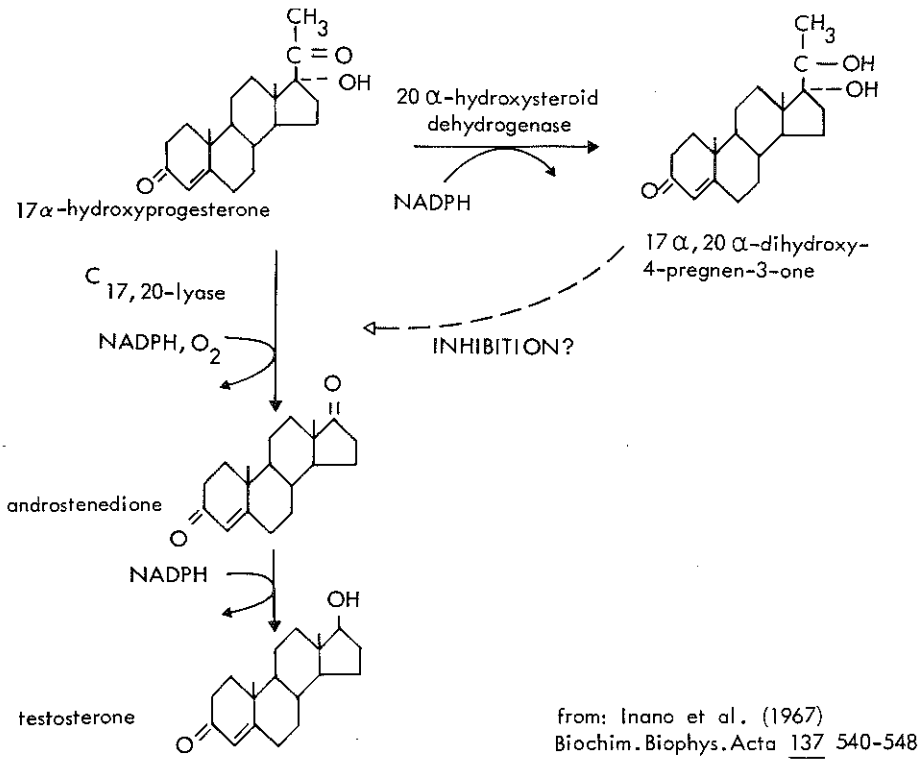


Fig. 1. A hypothetical mechanism for regulation of $C_{17,20}$-lyase activity and androgen biosynthesis.

thesis in the rat testis (fig. 1). This is a cytoplasmatic enzyme which converts 17α-hydroxyprogesterone to 17α,20α-dihydroxy-4-pregnen-3-one. The substrate specificity of the testicular enzyme differs from that of the 20α-hydroxysteroid dehydrogenases from the ovary, adrenal, placenta and liver, since progesterone is a poor substrate. The product 17α,20α-dihydroxy-4-pregnen-3-one was shown to be an inhibitor of the $C_{17,20}$-lyase. The K_m-value of this enzyme for 17α-hydroxyprogesterone was 1.8 μM, whereas the K_i-value for 17α,20α-dihydroxy-4-pregnen-3-one was 9 μM. Competition of the 20α-hydroxysteroid dehydrogenase with the $C_{17,20}$-

lyase for the substrate 17α-hydroxyprogesterone may also result in a decreased androgen production. 17α,20α-Dihydroxy-4-pregnen-3-one itself is not converted to androstenedione and testosterone. It is noteworthy that human testis can form 17α,20α-dihydroxy-4-pregnen-3-one from pregnenolone and 17α-hydroxypregnenolone[20,21], although in human testis the Δ^5,3β-hydroxysteroid pathway is of more importance than the Δ^4,3-oxosteroid pathway.

All experiments that have been reported in the literature were carried out with subcellular fractions of total testis tissue, and information on either the endogenous levels of inhibitor, or on the intercellular localization of the 20α-hydroxysteroid dehydrogenase was not available. Therefore, four different types of experiments were carried out:

1. The metabolism of radioactively labelled progesterone and 17α-hydroxyprogesterone and the formation of 17α, 20α-dihydroxy-4-pregnen-3-one were studied in preparations of rat and rabbit testis.
2. The effect of potential inhibitors on the $C_{17,20}$-lyase activity was investigated in <u>in vitro</u> incubation experiments and during rabbit testis perfusions.
3. A method for the estimation of 17α,20α-dihydroxy-4-pregnen-3-one was developed and it was used to measure the endogenous levels of the 17α,20α-dihydroxy-4-pregnen-3-one in testis and testicular venous blood.
4. The localization of 20α-hydroxysteroid dehydrogenase was studied using isolated interstitial tissue and seminiferous tubules from dissected rat testis.

Selective inhibition of $C_{17,20}$-lyase activity would depress testosterone biosynthesis without interfering with the biosynthesis of corticosteroids. This control of androgen production might be of potential interest in the treatment of benign prostatic hypertrophy, hirsutism and androgen-dependent tumors. Therefore, the effect of a new synthetic inhibitor of $C_{17,20}$-lyase, the 17β-ureido-1,4-androstanedien-3-one[22], was characterized.

1.1.3 A review

The enzymes involved in steroidogenesis and their steroid inhibitors are listed in table 1 at the end of this paragraph. This review is limited to the description of the direct effects of steroids on steroid biosynthesis and does not include observations which deal with indirect influences on steroid biosynthesis via feedback inhibition of gonadotrophins or ACTH secretion by the hypophysis.

Inhibition of cholesterol biosynthesis

The results of Haksar et al.[23,24] indicate that pregnenolone (400 μM)* inhibits sterol biosynthesis in bovine corpus luteum homogenates at a site before the formation of mevalonic acid. The incorporation of ^{14}C-acetate in progesterone by porcine corpora lutea in vitro can be decreased by several steroids (64 μM), the precise site at which inhibition occurs has not been determined (Cook et al.[25]). None of these steroids, however, could influence the progesterone synthesis from endogenous tissue sources. The relatively large tissue pools of cholesterol and cholesterol esters in testis and the uptake of cholesterol from blood[26,27] make it unlikely that inhibition of cholesterol biosynthesis is of importance for the regulation of steroidogenesis.

*When possible, the concentrations of inhibitors necessary for 50% inhibition, are given.

Inhibition of cholesterol side-chain cleavage

Pregnenolone (30 μM) has been shown to inhibit the side-chain cleavage of radioactive cholesterol in bovine corpus luteum[28,29], in bovine adrenal cortex[30] and in rat testis mitochondria[29]. Koritz[30,31] has proposed that ACTH increases mitochondrial permeability for pregnenolone, thereby lowering intramitochondrial pregnenolone concentrations and relieving the inhibition of steroidogenesis. However accumulation of pregnenolone, as caused by inhibition of Δ^5,3β-hydroxysteroid dehydrogenase in rat adrenal sections by the addition of cyanoketone does not result in inhibition of endogenous pregnenolone synthesis[32,33], suggesting that cholesterol side-chain cleavage is not significantly affected by large intracellular pregnenolone concentrations.

Testosterone was also found to be an inhibitor of the cholesterol side-chain cleaving activities in rat testis mitochondria *in vitro*[29,34] and in the rat adrenal gland *in vivo*[35]. Colby[35] reported that the rat adrenal side-chain cleaving activity was increased after orchiectomy and this effect could be reversed by testosterone administration. This probably reflects the influences of testosterone on mitochondrial cytochrome P_{450} levels[36] and on NADH-oxidase activity which will be described later.

The extent to which exogenous radioactive cholesterol enters the endogenous mitochondrial pool is unknown. Therefore formation of radioactive isocaproic acid or radioactive pregnenolone may not reflect changes in cholesterol side-chain cleaving activities. In light of this and the findings of Cook[25] and Farese[32], it may be concluded that the occurrence of inhibition of cholesterol side-chain cleavage[28-30,34,35] needs verification by measuring steroid production from endogenous sources.

Inhibition of 11β-hydroxylation

Corticosterone, when administered *in vivo*[37], or cortisol and deoxycorticosterone (10 µM), when added *in vitro*[38], inhibited the endogenous production of corticosterone by isolated rat adrenals. While studying this effect, Sauer[19] observed that NADH-oxidase was markedly inhibited and he suggested that interference with the electron transport chain decreased the rate of succinate-supported steroid 11β-hydroxylation by interfering with the reversed electron transport. NADH-oxidase inhibition by corticosterone (10 µM) has also been reported for pig heart sarcosomes[39,40].

Investigations by Sharma *et al.*[41] have drawn attention to the inhibiting influence of testosterone (10 µM) and androstenedione (10 µM) on the 11β-hydroxylation step *in vitro*. Testosterone (10 µM) and other androgenic steroids can also inhibit electron transfer at the site of NADH-oxidase in pig heart sarcosomes[40]. Treatment of rats with pharmacological doses of testosterone reduces corticosterone production and this is accompanied by a marked decrease in 11β-hydroxylase activities and mitochondrial cytochrome P_{450} levels in the adrenal gland (Colby and Brownie[36,42,43]). Orchiectomy results in an increased 11β-hydroxylase activity, which can be reversed by a testosterone therapy[35,44].

Burrow *et al.*[15] have shown that high concentrations of cortisol (1 mM) decrease the aldosterone production, probably by inhibiting adrenal protein synthesis[15-17]. However, Weinstein and Kliman[18] demonstrated more recently that lower doses (10 µM) of cortisol and 11-deoxycortisol had no effect on the biosynthesis of deoxycorticosterone, corticosterone, and aldosterone. The observation that high concentrations of testosterone (300 µM) blocked adrenal protein synthesis and the steroidogenic effect of ACTH is of some interest[16,17].

Therefore it may be concluded that low concentrations of certain steroids may interfere with electron transport

in the respiratory chain and very high concentrations with protein synthesis

Inhibition of 21-hydroxylation

Weinstein and Kliman demonstrated[18] that the addition of 17α-hydroxyprogesterone or 17α-hydroxypregnenolone (100 μM) to rat adrenal glomerulosa tissue decreases the endogenous production of deoxycorticosterone and corticosterone, resulting in a reduced *in vitro* production of aldosterone. The reduced production could be explained by an interference with the only hydroxylation step which the biosynthesis of all these steroids has in common, the 21-hydroxylation. Additional interference with 11β- or 18-hydroxylation, however, cannot be excluded. The 21-hydroxylation of pregnenolone and 17α-hydroxypregnenolone in the bovine adrenal gland can be inhibited by androstenedione, testosterone and dehydroepiandrosterone (20 μM)[45].

Inhibition of 17α-hydroxylation

The enzyme 17α-hydroxylase is inhibited by its product, 17α-hydroxyprogesterone (60 μM)[46]. This enzyme can also be inhibited by 20α-dihydroprogesterone and 17α,20α-dihydroxy-4-pregnen-3-one (Inano *et al.*[1]).

Inhibition of $C_{17,20}$-lyase

Huseby *et al.*[47] presented the first evidence indicating that progesterone may have an inhibiting effect on the $C_{17,20}$-lyase of mouse testis. Mahajan and Samuels[48] and others[13] reported a similar effect in rat testis homogena-

tes and more recently it was shown that dilution of the labelled substrate 17α-hydroxyprogesterone with non-labelled 17α-hydroxylated progesterone is not responsible for the competitive inhibition observed[49].

Neher and Kahnt[13] reported inhibition of the $C_{17,20}$-lyase by a number of steroids, e.g. pregnenolone and dehydroepiandrosterone inhibit the side-chain cleavage of 17α-hydroxypregnenolone and progesterone and 20α(and β)-dihydroprogesterone inhibit the side-chain cleavage of 17α-hydroxyprogesterone. The effects of 20α(and β)-dihydroprogesterone has been confirmed by Inano et al.[1]. In addition deoxycorticosterone and 17α,20α-dihydroxy-4-pregnen-3-one were found to be competitive inhibitors of this enzyme (see 1.1.2). The kinetics of the $C_{17,20}$-lyase in human adrenal glands were investigated in more detail by Jensen et al.[11,12]. The side-chain cleavage of 17α-hydroxypregnenolone (K_m = 0.4 μM) was noncompetitively inhibited by dehydroepiandrosterone (K_i = 5 μM), the tissue concentrations of the inhibitor were found to be in the order of 1.5 μM[50]. They postulated that this inhibition might contribute to the control of human adrenal dehydroepiandrosterone biosynthesis.

Inhibition of Δ^5,3β-hydroxysteroid dehydrogenase

The conversion of Δ^5,3β-hydroxysteroids to Δ^4,3-oxosteroids requires a Δ^5,3β-hydroxysteroid dehydrogenase and a Δ^5,Δ^4-isomerase. The overall rate of the reaction from Δ^5,3β-hydroxysteroids to Δ^4,3-oxosteroids estimates the rate of dehydrogenase reaction because the Δ^5,Δ^4-isomerase reaction is much faster[51]. Much of the knowledge of Δ^5,3β-hydroxysteroid dehydrogenase has been derived from experiments with the bacterial enzyme from Pseudomonas Testosteroni. Many inhibitors have been found (table 1). Two models for the regulation of Δ^5,3β-hydroxysteroid dehydrogenase

activity in vivo were proposed:

Studies of Wiener and Allen[9,10] have shown that the placental dehydrogenase (K_m for pregnenolone 2 μM) is non-competitively inhibited by progesterone (K_i = 10 μM) and 20α-dihydroprogesterone (K_i = 3 μM), that is at concentrations comparable to those found in the placenta (5-10 μM and 1 μM respectively).

The conversion of pregnenolone to progesterone by rat testis (K_m = 67 μM) was inhibited by 7α-hydroxyandrostenedione (K_i = 57 μM)[3]. Testicular 7α-hydroxylase activity, however, is low and is inhibited by 7α-hydroxyandrostenedione itself and by 7α-hydroxytestosterone. Data on tissue levels of 7α-hydroxyandrostenedione are not available.

Inhibition of Δ^5,Δ^4-steroid isomerase

Ewald[57] presented evidence for the existence of two distinct isomerases in bovine adrenal glands and demonstrated that the isomerization of 5-pregnene-3,20-dione, 17α-hydroxy-5-pregnene-3,20-dione and 5-androstene-3,17-dione can be inhibited by their corresponding Δ^5,3β-hydroxysteroids at concentrations of 30 μM[58]. Since the oxidation of 3β-hydroxysteroids is rate-limiting in the conversion of Δ^5,3β-hydroxysteroids to Δ^4,3-oxosteroids[51], inhibition of the isomerases is of little consequence for steroid biosynthesis.

Inhibition of 17β-hydroxysteroid dehydrogenase

The 17β-hydroxysteroid dehydrogenase, the enzyme involved in the last step in testosterone biosynthesis in the rat, can be inhibited by oestradiol (6 μM)[14] and by 7α-hydroxyandrostenedione (500 μM) but is activated by 7α-

hydroxytestosterone (500 μM)[3].

Inhibition of 20α-hydroxysteroid dehydrogenase

Oestradiol (K_i = 5 μM)[61], oestriol (K_i = 82 μM) and oestrone (K_i = 12 μM) were found to inhibit the 20α-reduction of progesterone (K_m = 470 μM) by the placental 20α-hydroxysteroid dehydrogenase and are thought to take part in the regulation of this enzyme activity[9,10].

Inhibition of steroid sulfatase

It was demonstrated by Notation and Ungar[4-6] and by Payne et al.[7,8] that a large number of steroid metabolites inhibit the steroid sulfatase activity of human and rat testis. However, it is not certain what role, if any, the steroid sulfates play in steroid hormone production - this will be discussed later (see part II). It is suggested that modulation of the sulfatase activity by free steroids may regulate the release of essential free steroid precursors of testosterone. The reported K_i-values are in the same range as the K_m-values for the different steroid sulfates (1-10 μM). The concentrations of dehydroepiandrosterone and its sulfate in human testis were recently estimated by Ruokonen[62] and are 0.03 μM and 1 μM respectively. It is difficult to conclude whether the larger concentration of the steroid sulfate is a result of inhibition of the sulfatase or, more likely, of a low sulfatase level when compared to 3β(and 17β)-hydroxysteroid dehydrogenase activities.

Miscellaneous inhibitions of steroidogenesis

Two other compounds, which may influence steroidogenesis through inhibition of the enzymes involved and which may have physiological significance are 3',5'-cAMP and some indoles.

The postulated intracellular mediator of trophic hormone action, 3',5'-cAMP stimulates pregnenolone formation[63]. In addition to this, 3',5'-cAMP at high concentrations can inhibit the Δ^5,3β-hydroxysteroid dehydrogenase in rat adrenal[64-66] and ovarian tissue[67]. The conclusion drawn from these studies is that 3',5'-cAMP can compete with NAD^+-dependent steroid enzymes for the available NAD^+ or that 3',5'-cAMP stimulates reactions that compete for NAD^+ [65,68]. The significance of this effect of 3',5'-cAMP in the control of steroidogenesis requires further clarification[66].

Recently Kinson[69] and Ellis[70] observed that melatonin and serotonin (2 μM), when added in vitro, can inhibit several steps of rat testicular androgen biosynthesis. These findings will undoubtedly stimulate further research on the significance of these indoles for the function of the testis.

Synthetic inhibitors of steroid biosynthesis have proved to be a useful tool for studying the effects of decreased steroid biosynthesis. A steroidal cyanoketone, for example, inhibits the Δ^5,3β-hydroxysteroid dehydrogenase and Δ^5,Δ^4-isomerase[71,72] and in high concentrations the cholesterol side-chain cleavage[73]. Administration of this compound to pregnant rats and mice can provide an experimental model for congenital adrenal hyperplasia[74] and other defects[75,76], due to a deficiency of Δ^5,3β-hydroxysteroid dehydrogenase. Experiments with isoxazol produce similar results[75,77]. Ovulation in immature rats can be prevented by this cyanoketone, apparently also a result of inhibited progesterone

biosynthesis[78].

Nonsteroidal inhibitors have been reviewed by Gaunt et al.[79] and by Dorfman and Ungar[80]. Further publications concerning their effects on testicular steroid biosynthesis are from Tamaoki and Shikita[81], Inano et al.[82], Bloch et al.[76] and Goldman[83].

table 1 INHIBITION OF STEROID BIOSYNTHESIS BY STEROIDS IN VITRO

enzyme (and source)	substrate	inhibitor	K_i (type of inhibition★)	References
cholesterol side-chain cleaving enzyme				
bovine corpus luteum	cholesterol (exogenous)	pregnenolone		28,29
bovine adrenal cortex	"	"		30
rat adrenal gland	"	testosterone (in vivo)		35
rat testis	"	pregnenolone		29
"	"	testosterone		29,34
11β-hydroxylase				
rat adrenal gland	endogenous precursors	corticosterone		37
"	"	deoxycorticosterone		38
"	deoxycorticosterone	cortisol		15
"	endogenous precursors	"		38
"	"	"	no inhibition	18
"	deoxycorticosterone	androstenedione	K_i=18 μM (comp.)	41
"	"	testosterone		41
"	"	" (in vivo)		35,36,42,43
21-hydroxylase				
rat adrenal (glomerulosa)	endogenous precursors	17α-hydroxyprogesterone		22
"	"	17α-hydroxypregnenolone		22
bovine adrenal cortex	progesterone	testosterone and		45
"	pregnenolone	dehydroepiandroste-		
"	17α-hydroxypregnenolone	rone		
17α-hydroxylase				
rat testis	progesterone	17α-hydroxyprogesterone		46
"	"	20α-dihydroprogesterone		1
"	"	17α,20α-dihydroxy-4-pregnen-3-one		1

table 1 (continued)

enzyme (and source)	substrate	inhibitor	K_i (type of inhibition★)	References
$C_{17,20}$-lyase				
mouse testis	17α-hydroxyprogesterone	progesterone		47
rat testis	"	progesterone	(comp.)	13,48,49
"	"	20α(β)-dihydroproges-terone		1,13
"	"	17α,20α-dihydroxy-4-pregnen-3-one	K_i= 9 μM(comp.)	1
"	"	deoxycorticosterone	K_i= 3 μM(comp.)	1
human adrenal	17α-hydroxypregnenolone	dehydroepiandroste-rone	K_i= 5 μM(comp.)	11,12
Δ^5,3β-hydroxysteroid dehydrogenase				
Pseudomonas Testosteroni	dehydroepiandrosterone	17α-hydroxyprogeste-rone	K_i=30 μM(comp.)	52
"	"	androstenedione	K_i=30 μM(comp.)	52
"	"	oestradiol	K_i= 2 μM(comp.)	52
bovine adrenal cortex	pregnenolone	17α-hydroxyprogeste-rone		53
"	"	progesterone		54
"	"	androstenedione	(comp.)	54
"	"	11β-hydroxyandroste-nedione		34
bovine corpus luteum	dehydroepiandrosterone	androstenedione		54
human placenta	pregnenolone	progesterone	K_i=10 μM(non-c.)	9,10
"	"	20α-dihydroprogeste-rone	K_i= 3 μM(non-c.)	9,10
"	"	20β-dihydroprogeste-rone	K_i= 4 μM	10
rat testis	dehydroepiandrosterone	oestradiol		14
"	pregnenolone	7α-hydroxyandroste-nedione	K_i=57 μM(comp.)	3
human testis	androstenediol	oestradiol		55

table 1 (continued)

enzyme (and source)	substrate	inhibitor	K_i (type of inhibition★)	References
Δ^5,Δ^4-isomerase				
bovine adrenal cortex	5-pregnene-3,20-dione	progesterone		56
"	5-androstene-3,17-dione	"		56
guinea pig adrenal gland	5-pregnene-3,20-dione	pregnenolone		57,58
"	17α-hydroxy-5-pregnene-3,20-dione	17α-hydroxypregnenolone		57,58
"	5-androstene-3,17-dione	dehydroepiandrosterone		57,58
17β-hydroxysteroid dehydrogenase				
rat testis	androstenedione	oestradiol		14
"	"	7α-hydroxyandrostenedione		3
guinea pig liver	testosterone	androstenedione	K_i=70 μM(comp.)	59
"	"	dehydroepisulfate		59
Pseudomonas Testosteroni	"	testosterone		60
20α-hydroxysteroid dehydrogenase				
human placenta	progesterone	oestriol	K_i=82 μM	9,10
"	"	oestrone	K_i=12 μM	9,10
"	"	oestradiol	K_i= 5 μM	61

table 1 (continued)

enzyme (and source)	substrate	inhibitor	K_i (type of inhibition★)	References
steroid sulfatase				
human testis	pregnenolone sulfate	pregnenolone		8
"	and dehydroepiandrosterone sulfate	20α-dihydroprogesterone		8
"	"	5-pregnene-3β,20α-diol	K_i=15 μM(comp.)	8
"	"	3β,21-dihydroxy-5-pregnen-3-one		8
"	"	5α-androstane-3α,17β-diol	K_i=40 μM(comp.)	8
rat testis	pregnenolone sulfate	testosterone		5,6
"	"	5-pregnene-3β,20α-diol	K_i= 3 μM(comp.)	5
"	"	17α-hydroxypregnenolone	K_i=14 μM(comp.)	5
"	"	pregnenolone	K_i= 8 μM	5
"	dehydroepiandrosterone sulfate	dehydroepiandrosterone	K_i= 8 μM(comp.)	5,6
"	"	5α-androstane-3α,17β-diol	K_i= 3 μM	7
"	"	5α-androstane-3β,17β-diol	K_i=1 μM(comp.)	5,7
"	"	testosterone	K_i=12 μM(comp.)	5,6,7
"	androstenediol sulfate	androstenediol		7
"	"	oestradiol		7
"	oestradiol sulfate	oestradiol	K_i=16 μM(comp.)	7
"	"	testosterone	K_i=13 μM	7

★ comp. = competitive
non-c.= non-competitive

1.2 Materials and methods

1.2.1 Materials

Solvents and reagents were obtained from UCB, Brussels and Merck, Darmstadt. Solvents were distilled before use. Diethylether was treated with a concentrated ferrosulphate solution prior to distillation and was used within a week. Toluene, when used for gas chromatography, was redistilled. NADPH, glucose-6-phosphate and the glucose-6-phosphate dehydrogenase were purchased from Boehringer, Mannheim. Bovine serum albumin was purchased from Povite Products, Amsterdam.

Steroids were purchased from Steraloids Inc., 20α-dihydroprogesterone from Ikapharm and the 17α,20α-dihydroxy-4-pregnen-3-one was a gift from the Steroid Reference Collection of the Medical Research Council, Great Britain, this steroid is now commercially available (Steraloids). 17β-Ureido-1,4-androstadien-3-one was obtained through the courtesy of Dr. G.E. Arth, Merck Sharpe and Dohme Research Laboratory, Rahway, U.S.A.. These steroids were used without further purification.

Radioactive steroids: 7α-^{3}H-17α-Hydroxyprogesterone (15 Ci/mM), 4-^{14}C-17α-hydroxyprogesterone (37 mCi/mM), 4-^{14}C-testosterone (58 mCi/mM), 1,2-^{3}H-testosterone (37 Ci/mM) and 4-^{14}C-androstenedione (60 mCi/mM) were obtained from Radiochemical Centre, Amersham and 1,2-^{3}H-progesterone (36 Ci/mM) and 4-^{14}C-progesterone (57 mCi/mM) from the New England Nuclear Corporation, Boston.

7α-^{3}H- and 4-^{14}C-Labelled 17α,20α-dihydroxy-4-pregnen-3-one were prepared from radioactive 17α-hydroxyprogesterone using an enzymatic reduction with a soluble rat testis

preparation (80 mg protein) and a NADPH regenerating system under nitrogen for 1 hour. After extraction the 17α,20α-dihydroxy-4-pregnen-3-one was purified by chromatography on paper in a Bush AII system for 16 hours, the yield exceeded 50%.

All radioactive steroids were purified by paper chromatography and thin-layer chromatography when necessary.

1.2.2 Techniques

Radioactivity was measured with a liquid scintillation counter (Packard model 3375 or Nuclear Chicago, Mark I) to an accuracy better than 1%. Quench corrections were usually calculated from external standard ratios. The samples were counted in a solution containing 4 g diphenyloxazole (PPO) and 40 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per litre or a modified Bray's solution[84]. When ^{3}H and ^{14}C were measured simultaneously, the $^{3}H/^{14}C$ ratio in the samples was kept within 1 and 100.

Chromatographic techniques

Paper chromatography was carried out on Whatman No. 20 paperstrips in a Bush AII system (petroleum ether:methanol:water = 100:70:30, by vol.) or a Bush BI system (petroleum ether:toluene:methanol:water = 25:25:35:15, by vol.)[85].

Thin-layer chromatography (t.l.c.) was performed on precoated silicagel plates (Merck F_{254}, 20x20 cm). Samples for gas chromatography were purified on t.l.c. plates which had been prerun in toluene:methanol = 9:1 (by vol.).

Detection of radioactive steroids on paper chromatograms was carried out with a Packard radiogramscanner (model 7200) and on t.l.c. plates with a Panax thin-layer scanner. Gas chromatography (g.l.c.) was carried out on a Hewlett-Packard Biomedical analyser, equipped with a tritium electron capture detector. The stationary phase con-

sisted of 1% QF-1 coated on Gas-Chrom Q (80-100 mesh, Applied Science) and argon (5% methane) was used as carrier gas.

Acetylations were carried out overnight in an acetic anhydride pyridine mixture (1:1).

Protein determinations were carried out as described by Lowry et al.[86].

1.2.3 Preparation of tissue fractions and incubations

Rats of the Wistar strain (weight 200-250 g) were killed by cervical fracture and subsequent bleeding. Rabbits (New Zealand White-strain, 2.5-3.5 kg) were anesthetized with Hypnorm (Philips-Duphar) and the testes were removed. After removal of the capsule the testes were homogenized in 0.25 M sucrose using a Potter Elvejhem homogenizer. For fractional centrifugation a Sorvall centrifuge (RC-2B) and a Beckmann ultracentrifuge (L2-65B, type 50 rotor) were used. The following fractions were prepared: the 800xg supernatant (15 min), the 800-105,000xg precipitate (60 min, combined microsomal and mitochondrial preparation) and the 105,000xg supernatant (60 min).

Incubations were carried out in Krebs-Ringer bicarbonate buffer (pH 7.4). The substrate 17α-hydroxyprogesterone, 7α-^{3}H-17α-hydroxyprogesterone (1-3x10^{6} dpm) and the steroid inhibitor were dissolved in methanol together with 1 µg Tween-20 in the incubation tube and the methanol was evaporated. The mixture was dissolved in a small volume of buffer, containing NADPH (1 mM), glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase. The reaction was started by adding the tissue preparations. The final volume was 1 to 1.5 ml. For some experiments the tissue was minced with scissors, and then approximately 100 mg was weighed

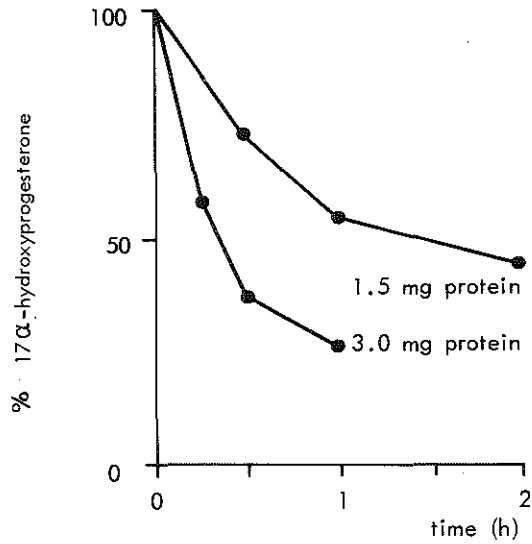


Fig. 2. Conversion of 17α-hydroxyprogesterone (3 μM) by a 800 x g supernatant of rabbit testis, plotted against the time. Each value is the mean of a duplicate estimation.

and transferred to an incubation tube, containing the incubation medium (plus 20 mM glucose). The incubation was started by addition of the substrate mixture. Rabbit testes were incubated at 37°C[87] but rat testes were incubated at 33°C, because of the lower scrotal temperature[88]. All incubations were carried out in a 95% oxygen, 5% carbondioxide atmosphere. After preliminary experiments an incubation time of 30 min was chosen for incubation experiments with rabbit testis (fig. 2) and 60 min for rat testis preparations.

1.2.4 Extractions and isolation of steroids

The incubation was stopped by the addition of 2-3 ml ethyl acetate containing 17α-hydroxyprogesterone, androstenedione, testosterone, 17α,20α-dihydroxy-4-pregnen-3-one

(50 µg each) plus 4-^{14}C-testosterone (10^4 dpm), 4-^{14}C-androstenedione (10^4 dpm), 4-^{14}C-17α-hydroxyprogesterone (5.10^3 dpm) and in some experiments 4-^{14}C-17α,20α-dihydroxy-4-pregnen-3-one (5.10^3 dpm). After extraction with 3x2 ml of ethyl acetate, the combined extracts were washed once with water and evaporated to dryness. Separation with paper chromatography (Bush AII) gave four fractions containing: 1) 17α,20α-dihydroxy-4-pregnen-3-one, 2) testosterone, 17α-hydroxyprogesterone and 5α-androstane-3α(β),17β-diol, 3) androstenedione and 3α(β)-hydroxy-5α-androstan-3-one. They were located on the paperstrip under u.v. light (254 nm) or by scanning for radioactivity. The steroids were eluted with 10 ml methanol or 10 ml methanol-water (9:1). Fraction 1 was further purified with paper chromatography (Bush BI). Fractions 2 and 3 were acetylated and subjected to respectively t.l.c. (toluene:ethyl acetate 1:4, by vol.) and paper chromatography (Bush AII). In some experiments a relatively small amount of 5α-androstanedione was found, as identified by its Rf-value in the Bush AII system and its non-acetylatable character. The isolated steroids (as acetates) were counted for ^{3}H and ^{14}C. The recovery from the isolation procedure was calculated from the ^{14}C content of the sample. These data were subsequently used to calculate the formation rate of the steroid product.

1.2.5 Perfusions

The technique for <u>in vitro</u> perfusion of rabbit testis is described in Materials and Methods of the second part of this thesis (paragraph 2.3.2). The same technique was used for the perfusion experiments with inhibitor. The substrate 1,2-^{3}H-progesterone was dissolved in approximately 4 ml of 0.15 M NaCl solution (5% albumin, 4 µg Tween-20) and was infused continuously into the testicular arterial blood at a constant rate, the doses varied from 10^6 to $3x10^6$ dpm ^{3}H

per 30 min. The inhibitor was introduced in the same way over a 20 min period, starting at a suitable time (paragraph 1.4.3). The testicular venous blood was collected in 30 min fractions. 4-^{14}C-Steroids (see above) were added to the plasma without carrier steroids. The steroids were isolated and counted as described for the incubation samples. Testosterone acetate was hydrolized (80% methanol in water, 1 M NaOH), purified by t.l.c. (toluene:ethyl acetate 2:1, by vol.), estimated by gas chromatography as the chloroacetate and counted for ^{3}H and ^{14}C.

1.2.6 Estimation of testosterone

Essentially the same method was used as described by Brownie et al.[89], except that 20α-dihydroprogesterone chloroacetate was used as an internal standard during g.l.c. analysis. Precoated t.l.c. plates were used and benzene was replaced by toluene throughout the method.

1.2.7 Estimation of 17α-hydroxyprogesterone

The competitive protein binding assay for the estimation of 17α-hydroxyprogesterone was similar to the method described by de Jong and van der Molen[90] for progesterone, replacing ^{3}H-progesterone by ^{3}H-17α-hydroxyprogesterone. The binding of ^{3}H-17α-hydroxyprogesterone decreased from 55 to 19% when 10 ng of unlabelled 17α-hydroxyprogesterone was added. Accuracy and precision are given in table 2.

Table 2 ACCURACY AND PRECISION OF 17α-HYDROXYPROGESTERONE ESTIMATION

amount added (ng)	amount estimated (ng)	S.D. (ng)	n
0	1.8	0.3	6
10	11.5	1.0	4
25	24.0	1.7	4
50	50	2	4

When analysing biological samples, three aliquots of different sizes were taken from the purified 17α-hydroxyprogesterone containing fraction and were subjected to a competitive protein binding assay. This fraction contains testosterone, which competes with ^{3}H-17α-hydroxyprogesterone for the binding protein. However, cross-reaction is low (4%); addition of 10 ng of testosterone to 1 ng of 17α-hydroxyprogesterone caused a decrease in binding from 48 to 43%, corresponding to 0.4 ng of 17α-hydroxyprogesterone. Nevertheless the purification step on t.l.c. prior to the assay was repeated once in order to achieve a good separation from testosterone.

1.2.8 Estimation of 17α,20α-dihydroxy-4-pregnen-3-one

The observation, that the 17α,20α-dihydroxy-4-pregnen-3-one can be oxidized with periodic acid to androstenedione[91,92], was used as a starting point for estimation. The method is summarized in table 3. The high sensitivity of electron capture detection allows accurate detection of the testosterone chloroacetate.

Table 3 ESTIMATION OF 17α,20α-DIHYDROXY-4-PREGNEN-3-ONE

1. Addition of 7α-^{3}H-17α,20α-dihydroxy-4-pregnen-3-one to plasma sample or acetone extract of tissue.
2. Defatting of the tissue extracts.
3. Alkaline ether extraction.
4. Paper chromatography (Bush BI), followed by t.l.c.
5. Oxidation with HIO_4 to androstenedione, t.l.c.
6. Enzymatic reduction of the 17-oxo group and conversion to testosterone chloroacetate, t.l.c.
7. - Sampling for counting of tritium
   - Sampling for mass determination by gas chromatography with electron capture detection.

Tissue extractions

Testes of rats and rabbits were collected as described and were frozen in liquid nitrogen when not processed immediately. Testes were homogenized in 15 ml acetone (Ultra-Turrax) and to each sample approximately 50,000 dpm of 7α-^{3}H-17α,20α-dihydroxy-4-pregnen-3-one (15 Ci/mM) (and 5,000 dpm 7α-^{3}H-17α-hydroxyprogesterone) was added. The homogenates were centrifuged and the pellets were resuspended in 10 ml ethanol-aceton (1:1, by vol.) and recentrifuged. This was repeated once and the combined extracts were concentrated under reduced pressure in a rotating evaporator to a volume of 2-3 ml. After transferring the extract to a centrifuge tube, warm methanol was added until a 70% methanolic solution was obtained. Defatting was achieved by freezing at minus 25°C followed by centrifugation. The solution was again concentrated to the water phase and extracted with diethylether (4x10 ml). The combined extracts were washed twice with water (5 ml) and the ether was evaporated.

Plasma samples

After addition of the 7α-^{3}H-17α,20α-dihydroxy-4-pregnen-3-one, the plasma samples were subjected to the alkaline ether extraction procedure.

Estimation method

The extract was chromatographed on paper in a Bush BI system. The steroid was located by scanning for radioactivity and then eluted with 10 ml 90% methanol (the 17α-hydroxyprogesterone containing fraction was also eluted and estimated with the competitive protein binding assay). The eluate was concentrated and further purified with t.l.c. (toluene:ethyl acetate 2:1). The 17α,20α-dihydroxy-4-pregnen-3-one fraction, eluted by methanol was concentrated to a volume of 1 ml. Oxidation was performed by addition of 0.5 ml acetic acid and 1.0 ml of 1% periodic acid solution (Riedel-Haen). After 1 h in the dark 8 ml of ethyl acetate was added and after mixing the solution was washed with 1x2 ml water, 2x2 ml of a 8% sodium bicarbonate solution and again with 1x2 ml of water. The ethyl acetate was evaporated to dryness and the residue was purified with t.l.c. (toluene:ethanol = 9:1, by vol.). The androstenedione containing region was scraped off and eluted with toluene. This fraction was reduced to testosterone by 100 µg sodium borohydride in 0.5 ml ethanol (2 h at 4°C). In later experiments the reduction was carried out by incubation with a hydroxysteroid dehydrogenase preparation (Worthington)[93]: Androstenedione and 2 µg of Tween-20 were dissolved in 1 ml Krebs-Ringer bicarbonate buffer (pH 7.4) containing NADH (50 mM) and 0.1 U. of the enzyme was added. After five minutes of incubation at room temperature, extraction by ethyl acetate was carried out. The extracts were concentrated to dryness, chloroacetylated, estimated by gas chromatography and counted for ^{3}H-radioactivity. The amount of 17α,20α-dihydroxy-4-pregnen-3-one was calculated with aid of the recovery of the ^{3}H-label.

1.2.9 Localization of the 20α-hydroxysteroid dehydrogenase

Dissection procedure

Interstitial tissue and seminiferous tubules of two rat testes were separated using the wet dissection technique of Christensen and Mason[94]. The isolated tissues were washed 2-3 times in a small volume of Krebs-Ringer bicarbonate buffer (pH 7.4) in order to remove the soluble 20α-hydroxysteroid dehydrogenase activity which was leaked from broken cells. Tissue fragments were recollected through centrifugation at approximately 300xg for 1 min. The tubular fraction was homogenized by a Potter Elvejhem homogenizer in 10 ml 0.1 M phosphate buffer (pH 7.0) containing 5 mM EDTA. The interstitial tissue was homogenized in 1 ml of phosphate buffer. Samples of the total homogenates were removed and used for protein determinations. Sonicated samples were assayed for phenyl esterase activity by measuring the rate of hydrolysis of p -nitrophenylacetate in 0.1 M Tris-HCl buffer (pH 8.0)[95].

The total homogenate was centrifuged at 10,000xg for 15 min, the supernatant was taken and centrifuged at 105,000xg for 60 min. The 105,000xg supernatant was assayed for 20α-hydroxysteroid dehydrogenase activity and protein.

20α-Hydroxysteroid dehydrogenase

The preparations were incubated in 0.1 M phosphate buffer (pH 7.0) containing 1 mM NADPH, 10 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The tubular fraction (2.8 mg protein/incubation) was incubated in 2 ml of buffer and the interstitial tissue fraction (0.18 mg protein/incubation) in 0.5 ml volume. Incubation was started by introduction of the substrate 17α-hydroxyprogesterone (approximately 40,000 dpm 4-^{14}C-17α-hydroxyproges-

terone, final concentration varied from 1-60 μM) and 30 respectively 60 min later the reaction was stopped by addition of ethyl acetate. Carrier steroids and 10,000 dpm 7α-^{3}H-17α,20α-dihydroxy-4-pregnen-3-one were added and the product was isolated as described in paragraph 1.2.2. The net conversion was calculated from the ^{3}H and ^{14}C content of the purified product. The velocity was constant for 60 min and a linear relationship was found between velocity and substrate concentration in both preparations. Enzyme activities were calculated on basis of the conversion at 60 μM substrate. The K_m-value of the partially purified enzyme was reported as 65 μM^2. Higher substrate concentrations resulted in decreased percentages of ^{14}C conversion and inaccurate measurements of radioactivity.

1.3 Metabolism of progesterone and 17α-hydroxyproges terone and the formation of 17α,20α-dihydroxy-4-pregnen-3-one

1.3.1 Metabolism of progesterone and 17α-hydroxyprogesterone

It is well established that testicular tissue can convert progesterone and 17α-hydroxyprogesterone to androstenedione and testosterone[96,98,99]. When using these substrates, other products are formed in addition to the steroids known to be involved in androgen biosynthesis. For example, the formation of 5α-reduced steroids, 3α(β)-hydroxysteroids and 20α(β)-hydroxysteroids has been reported[100,101].

The possible pathways for the metabolism of 17α-hydroxyprogesterone are given in fig. 3. In order to obtain a correct estimate of the $C_{17,20}$-lyase activities in rat and rabbit testes, it was necessary to identify the main $C_{17,20}$-lyase products formed in our incubation and perfusion experiments. In addition, the formation of the potential inhibitor 17α,20α-dihydroxy-4-pregnen-3-one under several experimental conditions was investigated.

Identification of metabolites

Five different metabolites were isolated after incubation of homogenates of rat testes with 7α-^{3}H-17α-hydroxyprogesterone. In addition to labelled androstenedione and testosterone, the presence of labelled 3(αorβ)-hydroxy-5α-androstane-17-one, 5α-androstane-3(αorβ),17β-diol and 17α,20α-dihydroxy-4-pregnen-3-one was established.

The identification of these steroids was based on similar chromatographic behaviour of the free steroids, their acetates and their oxidation products, and that of reference compounds. The two 3-hydroxysteroids were expected as

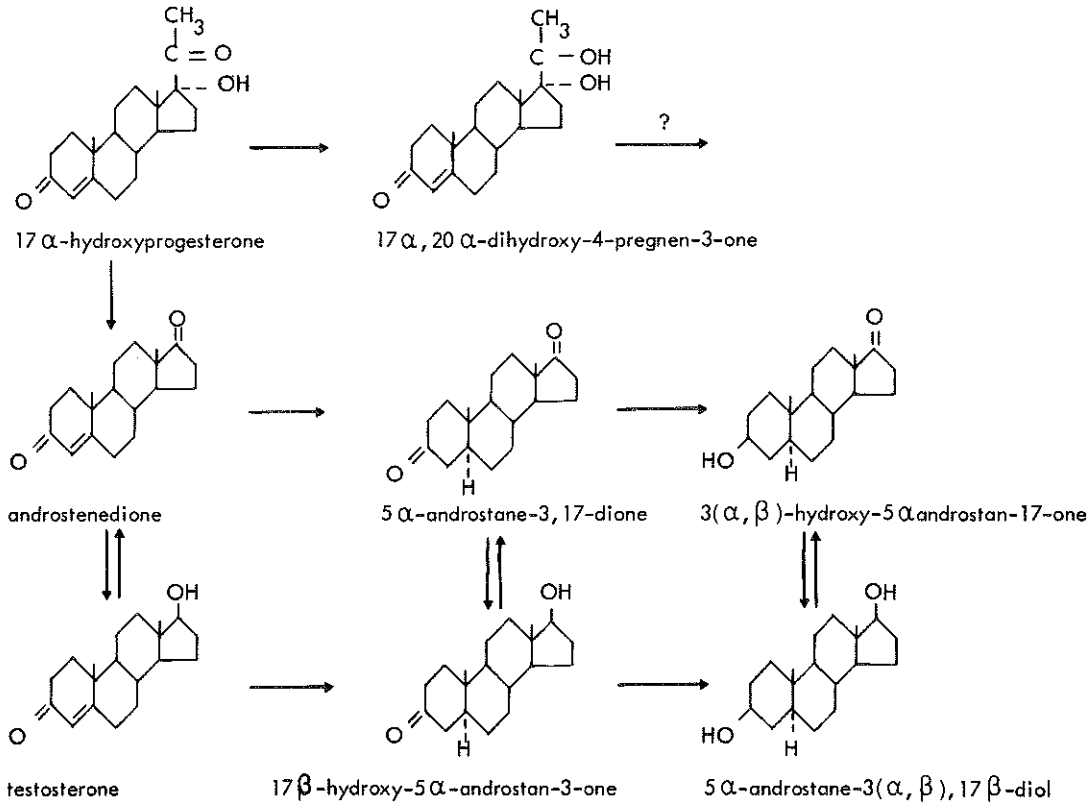


Fig. 3. Some theoretical possibilities for metabolism of 17α-hydroxyprogesterone in the testis.

products because the formation of these metabolites has been previously described[100,102]. Recently this work was confirmed by others[103-110]. When the incubation period was limited to 30 or 60 minutes, the contribution of these two metabolites to the total of $C_{17,20}$-lyase products was less than 3% and could usually be neglected. Therefore the sum of androstenedione and testosterone reflects $C_{17,20}$-lyase activity. The 17α,20α-dihydroxy-4-pregnen-3-one was also isolated and identified, thus confirming previous reports[101,111].

Incubation of rabbit testis homogenates with 7α-^{3}H-17α-hydroxyprogesterone revealed the formation of labelled 3-hydroxysteroids and 5α-androstane-3,17-dione as by-products of androstenedione and testosterone. After isolation of the labelled 3-hydroxysteroids, samples were recrystallized with eight different C_{19}-steroids and the presence of 3β-hydroxy-5α-androstane-17-one and 5α-androstane-3β,17β-diol was established (tables 4 and 5). The 5α-androstane-3,17-dione fraction was not fully characterized. After a shorter incubation period (30 min) only the 3β-hydroxy-5α-androstane-17-one contributed significantly to the total of $C_{17,20}$-lyase products. Rabbit testis also demonstrated 20α-hydroxysteroid dehydrogenase activity and the identity of

Table 4 RECRYSTALLIZATION OF 3β-HYDROXY-5α-ANDROSTAN-17-ONE, isolated from incubations of rabbit testis with 7α-^{3}H-17α-hydroxyprogesterone. After isolation, the ^{3}H-labelled metabolite was recrystallized with 50 mg of carrier steroid

Recrystallized from	Specific activity as 10^{-3} x dpm/mg			
	expt. 1		expt. 2	
	crystals	mother-liquor	crystals	mother-liquor
1. toluene/methylcyclohexane	32.4	55.1	30.5	45.0
2. dioxane/cyclohexane	32.3	32.4	30.2	30.3
3. methanol/water	31.2	31.4	29.7	-

17α,20α-dihydroxy-4-pregnen-3-one was confirmed by recrystallization with the authentic steroid (table 6). Table 7 summarizes the metabolic products of 17α-hydroxyprogesterone.

Table 5 RECRYSTALLIZATION OF 5α-ANDROSTANE-3β,17β-DIOL (see legend table 4)

Recrystallized from	Specific activity as 10^{-3} x dpm/mg	
	crystals	mother-liquor
1. toluene/methylcyclohexane	9.16	9.92
2. dioxane/cyclohexane	9.39	-
3. methanol/water	9.50	-

Table 6 RECRYSTALLIZATION OF 17α,20α-DIHYDROXY-4-PREGNEN-3-ONE (see legend table 4)

Recrystallized from	Specific activity as 10^{-3} x dpm/mg		
	expt. 1		expt. 2
	crystals	mother-liquor	crystals
1. methanol/cyclohexane	6.66	7.66	7.06
2. toluene/cyclohexane	6.67	-	7.22
3. toluene	6.47	-	6.84

Table 7 STEROID METABOLITES isolated after incubations of 7α-^{3}H-17α-hydroxyprogesterone with total testis homogenates

RAT	RABBIT
17α,20α-dihydroxy-4-pregnen-3-one	17α,20α-dihydroxy-4-pregnen-3-one
androstenedione	androstenedione
testosterone	testosterone
3(α,β)-hydroxy-5 -androstane-17-one	3β-hydroxy-5α-androstane-17-one
5α-androstane-3(α,β),17β-diol	5α-androstane-3β,17β-diol
	5α-androstane-3,17-dione

1.3.2 Formation of 17α,20α-dihydroxy-4-pregnen-3-one

During incubations of both rat and rabbit testes minces and broken cell preparations 7α-^{3}H-17α-hydroxyprogesterone was converted to labelled 17α,20α-dihydroxy-4-pregnen-3-one. The quantities of this potential inhibitor isolated, demonstrate that this is a major metabolite (table 8). It was found that the largest amounts of 17α,20α-dihydroxy-4-pregnen-3-one were in the soluble fraction, thus indicating that the 20α-hydroxysteroid dehydrogenase from rabbit testis is a soluble enzyme, which is in agreement with previous reports for the rat testis[1,2]. The activity present in the combined microsomal/mitochondrial fractions (800-105,000xg

Table 8 FORMATION OF ^{3}H-17α,20α-DIHYDROXY-4-PREGNEN-3-ONE (17,20-P). The conversion of ^{3}H-17α-hydroxyprogesterone (3 μM) to 17,20-P and lyase products during incubation with several testis preparations is expressed as a percentage of the total amount of tritium per incubation (each tissue fraction was equivalent to 100 mg of total testis tissue, wet weight).

*not corrected for losses during the isolation procedure

exp.	animal	preparation	% ^{3}H in 17,20-P	% ^{3}H in lyase products
1	rat	800xg supernatant	15*	58
		800-105,000xg pellet	4*	54
		105,000xg supernatant	23*	0.3
2	rat	800xg supernatant	10	70
		minced testis	9	64
3	rabbit	800xg supernatant	24*	10
		800-105,000xg pellet	2*	21
		105,000xg supernatant	25*	0.2
4	rabbit	800xg supernatant	15	17
		minced testis	6	14

pellet) can at least in part be explained by a contamination with the soluble fraction, because they were not washed or recentrifuged.

In the rabbit testis incubations, the amounts of $C_{17,20}$-lyase products, formed by the 800xg supernatant is half of that formed by the 800-105,000xg pellet. It is not likely, that the 17α,20α-dihydroxy-4-pregnen-3-one, formed by the cytosol, is responsible for the decreased $C_{17,20}$-lyase activity, since the final concentration of inhibitor was only 0.7 μM, which is too low to cause considerable inhibition (see paragraph 1.4.1).

When rabbit testis in vitro was continuously perfused with 7α-^{3}H-progesterone, labelled 17α,20α-dihydroxy-4-pregnen-3-one was detected in the venous effluent (table 9). The figures shown for this metabolite, in contrast to those shown for testosterone, have not been corrected for losses during the isolation procedures, therefore the amounts produced were approximately twice as high as the figures suggest. The amount of tritium incorporated into testosterone was found to be five to ten times higher than that incorporated into androstenedione. Thus the values for testostero-

Table 9 SECRETION OF ^{3}H-17α,20α-DIHYDROXY-4-PREGNEN-3-ONE (17,20-P) AND ^{3}H-TESTOSTERONE (T) BY RABBIT TESTES. ^{3}H-Steroids were isolated from the venous effluents after in vitro perfusions of rabbit testes with 7α-^{3}H-progesterone. Results are expressed as percentage ^{3}H of the total 7α-^{3}H-progesterone used. ^{3}H-17,20-P-values were not corrected for losses during the isolation procedure

testis	% ^{3}H-17,20-P	% ^{3}H-T
1	1.5	4.7
2	1.0	2.2
3	1.6	8.0
4	2.4	15
5	0.8	8.9

ne alone reflect the activity of the $C_{17,20}$-lyase. The data indicate that substantial amounts of ^{3}H-17α,20α-dihydroxy-4-pregnen-3-one were formed and secreted.

1.3.3 Kinetics of $C_{17,20}$-lyase from rabbit testis

From the Lineweaver-Burk plot (fig. 4) it can be concluded that the apparent K_m-value of the rabbit $C_{17,20}$-lyase for 17α-hydroxyprogesterone is 5 μM. This value is in the same range as that for the rat testis enzyme (1.8 μM)[1]. At high substrate concentrations, when enzyme saturation should be obtained, the percentage conversion of the added radioactive 17α-hydroxyprogesterone decreased to such low values that the estimations became inaccurate. Therefore, the substrate concentrations were kept at 3-6 μM in most of the experiments and incubations were not performed under conditions of enzyme saturation.

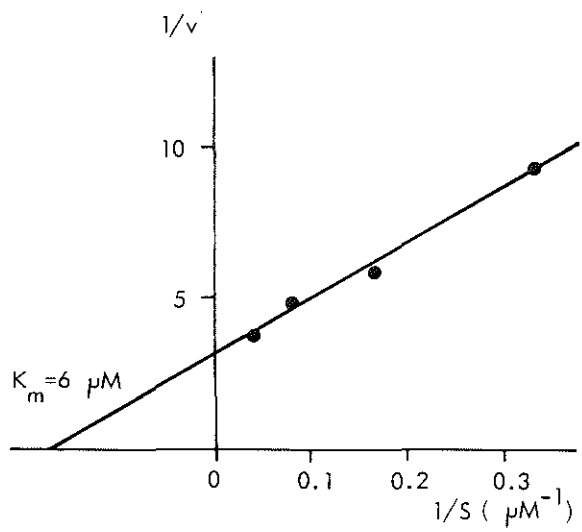


Fig. 4. Lineweaver-Burk plot of $C_{17,20}$-lyase activity for the conversion of 17α-hydroxyprogesterone to androstenedione in a 800 - 105,000 x g pellet from rabbit testis. Velocity (v) is expressed as nmoles of androstenedione and testosterone formed per mg of protein per 30 minutes (2.0 mg of protein per incubation.)

1.3.4 Discussion and conclusions

Androstenedione and testosterone are the main metabolites formed from 17α-hydroxyprogesterone metabolism in rat and rabbit testes. The presence of 5α-reductase activity in rat testis was first described by Inano and Tamaoki[100] and by Nayfeh and Baggett[101] and in 1972 by Folman et al.[105]. One of the products in rabbit testis, the 3β-hydroxy-5α-androstan-17-one has previously only been reported in rat testis[100,104]. Payne and Jaffe[109] obtained an indication for the formation of the 5α-androstane-3β,17β-diol although good evidence for its identification was not given.

Significant amounts of 3α-hydroxysteroids could not be detected after incubation with rabbit testis. Hydroxylation of androgens at the 7α-position[112] was not detectable because this eliminates the tritium label from the steroid molecule. The rate of 7α-hydroxylation is in any case low[3].

The results also seem to indicate that the 20α-hydroxysteroid dehydrogenase activity, relative to the $C_{17,20}$-lyase activity, is higher in rabbit than in rat testis, although the lack of enzyme saturation with substrate prevents drawing definite conclusions.

On basis of these experiments with labelled precursors it is concluded that 17α,20α-dihydroxy-4-pregnen-3-one is most probably formed under <u>in vivo</u> conditions.

1.4 Inhibition of $C_{17,20}$-lyase

1.4.1 Introduction

The enzyme $C_{17,20}$-lyase has a key role in the conversion of C_{21}-steroids to androgens. In rat testis this enzyme catalyses the rate-limiting step in the conversion of pregnenolone to testosterone[133]. The activity of this enzyme may be regulated by 17α,20α-dihydroxy-4-pregnen-3-one, as postulated by Inano and coworkers[1,2] (see paragraph 1.1.2) and also by 20α(and β)-dihydroprogesterone[1,13]. In vitro incubation experiments with subcellular fractions indicate that 17α,20α-dihydroxy-4-pregnen-3-one but not 20α(and β)-dihydroprogesterone are formed in this tissue. It was decided to investigate under several experimental conditions the inhibition of $C_{17,20}$-lyase. In addition to the incubation experiments, rabbit testes were perfused in order to study the possible effects of inhibitors on the steroid biosynthesis in the intact testis under more physiological conditions.

One of the inhibitors investigated was the new synthetic steroid, 17β-ureido-1,4-androstadien-3-one, which was selected by Arth et al.[22] from a series of androstane derivates as one of the best inhibitors for the $C_{17,20}$-lyase. These authors measured inhibition in terms of a decreased production of ^{14}C-acetate obtained by side-chain cleavage of 21-^{14}C-17α-hydroxyprogesterone. This inhibitor was further characterized and also tested in rabbit testis. The rabbit testis probably provides a better model for comparison with human testicular testosterone biosynthesis than the rat testis because of the predominance of the Δ^5,3β-hydroxysteroid pathway (see paragraphs 2.1 and 2.3.3).

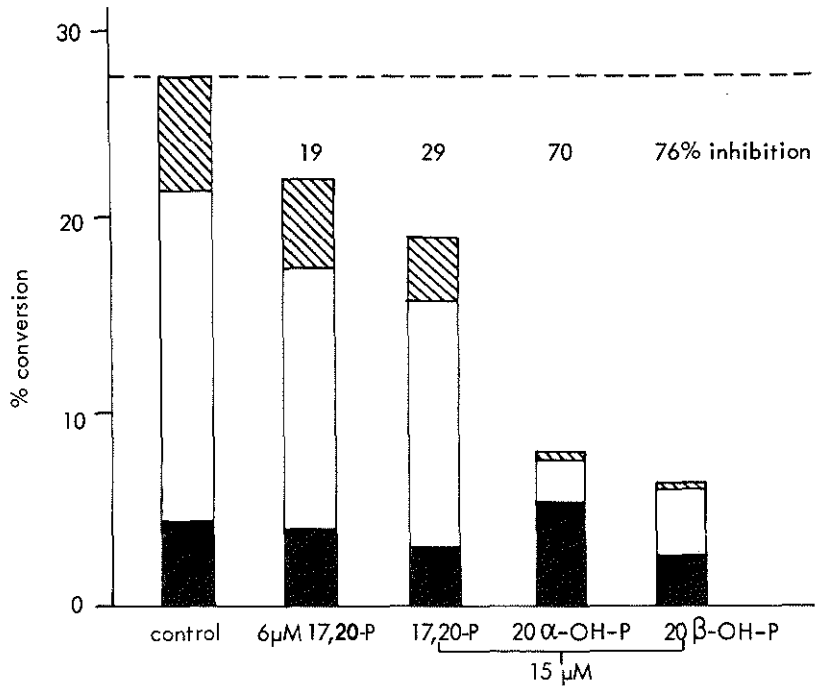


Fig. 5. Inhibition of $C_{17,20}$-lyase activity by 17α,20α-dihydroxy-4-pregnen-3-one (17,20-P), 20α-dihydroprogesterone (20α-OH-P) and 20β-dihydroprogesterone (20β-OH-P) in a 800 - 105,000 x g fraction from rabbit testis (1.8 mg of protein per incubation). 17α-Hydroxyprogesterone (3 μM) was used as a substrate. The vertical bars represent the sum of $C_{17,20}$-lyase products, the percentages of inhibition are given in the top of the figure. Each value is the mean of duplicate estimations. The rate of conversion without inhibitors was 0.45 nmoles per mg of protein per 30 min.

3β-hydroxy-5α-androstane-3-one testosterone androstenedione

1.4.2 Incubation experiments

A comparison was made between the effects of 20α(and β)-dihydroprogesterone and 17α,20α-dihydroxy-4-pregnen-3-one in their ability to inhibit $C_{17,20}$-lyase activity in rabbit testis in vitro (fig. 5). The 20-reduced metabolites of progesterone were found to be the better inhibitors.

When these compounds were added, the sum of $C_{17,20}$-lyase products decreased but the contribution of androstenedione to the products increased relatively to that of testosterone and 3β-hydroxy-5α-androstan-17-one. The latter is in accordance with inhibition of the $C_{17,20}$-lyase.

From comparison of the results in fig. 5 with that of table 8 (see paragraph 1.3.2) it can be concluded that the concentration of 17α,20α-dihydroxy-4-pregnen-3-one, necessary for inhibition (15 μM), was much higher than the amount formed during incubation (0.7 μM, from table 8) under comparable experimental conditions.

Investigation of the kinetic properties of the inhibition revealed that 20α-dihydroprogesterone is a competitive inhibitor with an apparent K_i-value of 2-5 μM (fig. 6). The corresponding value for 17α,20α-dihydroxy-4-pregnen-3-one, also a competitive inhibitor, was 9 μM[1]. On the basis of

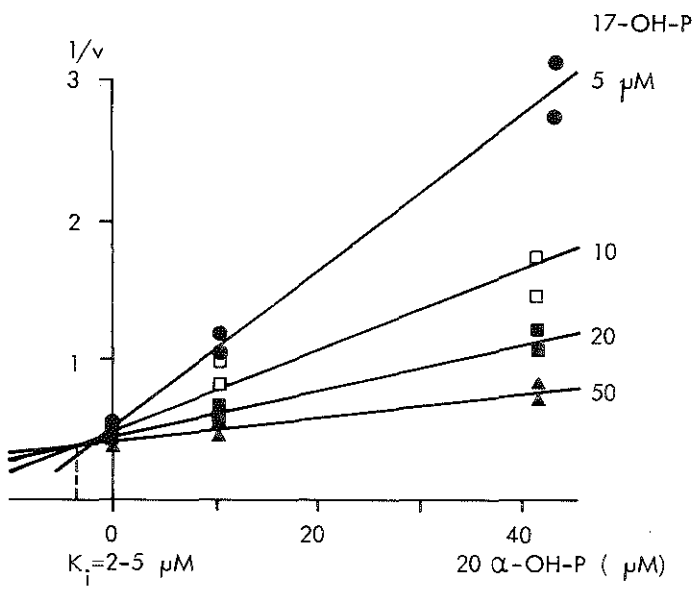


Fig. 6. Inhibition of the $C_{17,20}$-lyase by 20α-dihydroprogesterone (20α-OH-P) in a washed 900 - 105,000 x g fraction of rat testis (1.3 mg of protein per incubation), represented in a Dixon type plot[97]. 17α-Hydroxyprogesterone (17-OH-P) was used as a substrate. The velocity is given as nmoles of androstenedione and testosterone formed per mg of protein per h.

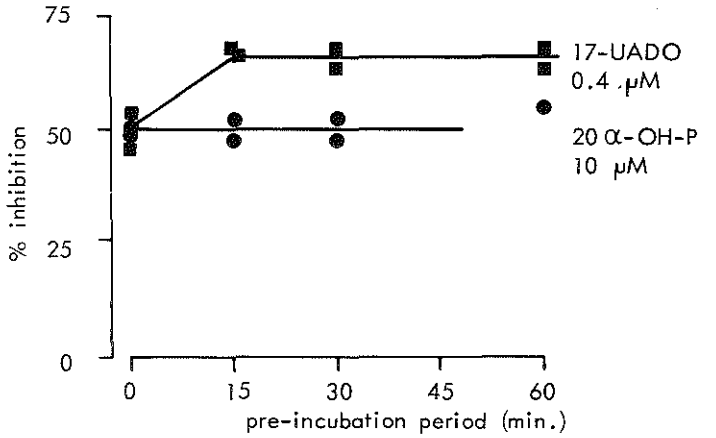


Fig. 7. The effect of preincubation with inhibitor on the inhibition of $C_{17,20}$-lyase in minced rat testis. Decapsulated, minced testis tissue (9.3 mg of protein per incubation) was preincubated with 20 α-dihydroprogesterone (20 α-OH-P) or with 17 β-ureido-1,4-androstadien-3-one (17-UADO) for various time periods. In control experiments the tissue was preincubated without inhibitor. The reactions were started by adding the substrate 7α-^{3}H-17α-hydroxyprogesterone (final concentration 5 μM). The rate of conversion in the control incubations was 0.18 nmoles of androstenedione and testosterone formed per mg of protein per h.

these similarities we have also used 20α-dihydroprogesterone as an inhibitor in the perfusion experiments.

The next experiments were designed to find out if the inhibitors can enter rapidly into the testicular cells (fig. 7). If the inhibitor penetrated slowly when compared with the substrate 17α-hydroxyprogesterone, preincubation with inhibitor should result in a better inhibition. From fig. 7 it can be concluded that only for the 17β-ureido-1,4-androstadien-3-one the rate of penetration does influence the degree of inhibition.

Experiments were carried out to determine the influence of albumin on enzyme activity and inhibition (table 10). In solution albumin (4%) binds in the order of 90% of the common Δ^4,3-oxosteroids[113] and the (tenfold) decrease in free substrate and inhibitor concentrations may explain the decreased conversion found, as well as the less efficient inhibition by 20α-dihydroprogesterone (table 10). Billiar and

Little[114,115] also observed that human albumin (2%) decreased the 20α-hydroxysteroid dehydrogenase activity of a placental enzyme preparation, but low concentrations (0.2%) increased the activity, apparently due to inactivation of a lipophilic, inhibiting substance. A recent paper from Hamilton et al.[116] demonstrated the diversity and species differences in the action of several plasma proteins on steroid 21-hydroxylase, Δ^5,3β-hydroxysteroid dehydrogenase and Δ^5,Δ^4-isomerase from adrenal origin.

Table 10 EFFECT OF ALBUMIN ON THE $C_{17,20}$-LYASE ACTIVITY AND THE INHIBITION BY 20α-DIHYDROPROGESTERONE (20α-OH-P).
Incubation with the 800-105,000xg fraction of rabbit testis (2.0 mg protein per incubation). Conversion of 7α-^{3}H-17α-hydroxyprogesterone (4 μM) is expressed as the total percentage ^{3}H present in the $C_{17,20}$-lyase products. The control rate of conversion (without albumin) was 1.5 nmoles per mg of protein per 30 min

bovine serum albumin added	% ^{3}H in $C_{17,20}$-lyase products		% inhibition by 20α-OH-P
	control	20α-OH-P (10 μM)	
-	48	23	49
	47	25	
4%	31	25	17
	30	25	

1.4.3 Perfusion experiments with rabbit testis

Continuous infusion with tracer amounts of ^{3}H-labelled progesterone was carried out and the incorporation of label into the $C_{17,20}$-lyase products was estimated. This in order to determine whether or not an infused inhibitor had a specific effect on the $C_{17,20}$-lyase activity when infused over a certain period. As mentioned before (paragraph 1.3.2) the amount of ^{3}H-testosterone secreted was about five to ten times more than that of the ^{3}H-androstenedione, thus the values for ^{3}H-testosterone alone are given. The tritium incorporation is expressed as a percentage of the total tritium in the venous blood sample, this in order to correct for possible small fluctuations in the flow rate and the infusion rate of labelled progesterone.

During infusion through the testis labelled progesterone was converted to 17α-hydroxyprogesterone, the latter being the actual substrate for the $C_{17,20}$-lyase. An equilibrium with the endogenous steroid production was apparently reached after two hours perfusion because the secreted testosterone had a constant specific activity after this time (fig. 8). Therefore, in subsequent experiments the potential inhibitors were infused after two hours perfusion. The final concentration of the inhibitor in the perfusion medium during the period of infusion was in the order of 10 μM (except for the third perfusion). Inhibition would result in decreased ^{3}H-testosterone secretion and might enhance the ^{3}H-17α-hydroxyprogesterone secretion.

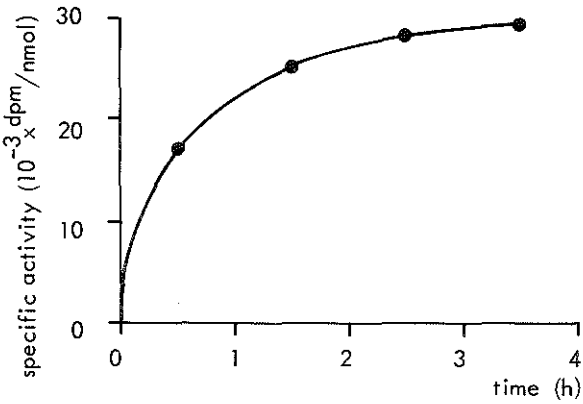


Fig. 8. Specific radioactivity of testosterone isolated from the testicular venous blood after in vitro perfusion of rabbit testis with 7α-^{3}H-progesterone (2.3 x 10^{6} dpm and 30 pmol per h).

From fig. 9 it can be concluded that 17α,20α-dihydroxy-4-pregnen-3-one did not influence the ^{3}H-incorporation into testosterone at the time of infusion or later. During the second perfusion 4-^{14}C labelled inhibitor was infused and the larger part of it appeared immediately into the venous blood, only 0.6% (uncorrected) of the inhibitor could be extracted from the testis tissue (fig. 10). In perfusion number 3 the same amount of 17α,20α-dihydroxy-4-pregnen-3-one was administered over a longer period, apparently also without effect.

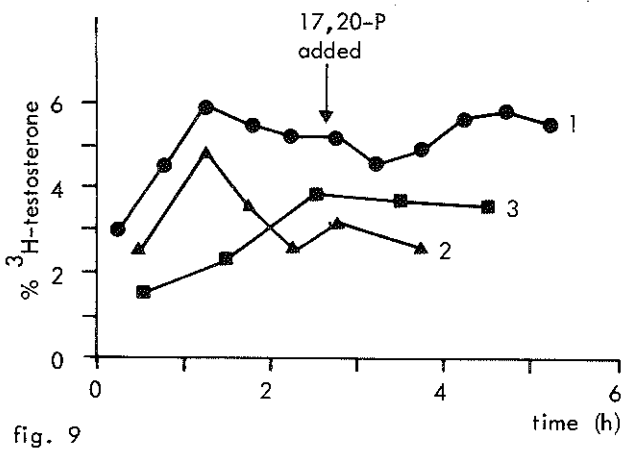


fig. 9

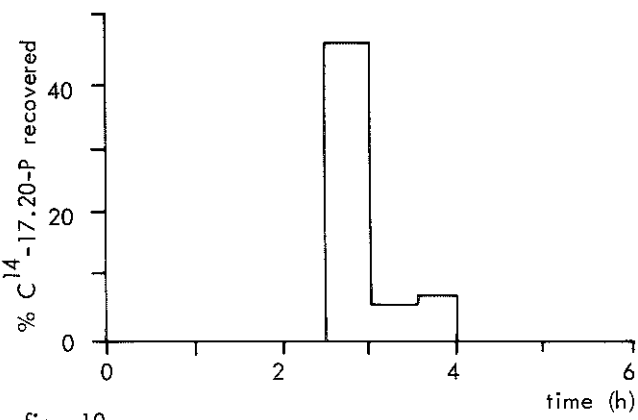


fig. 10

Fig. 9. ^{3}H-Testosterone, isolated from the testicular venous blood after in vitro perfusion of rabbit testis with 7α-^{3}H-progesterone. ^{3}H-Testosterone values are expressed as percentage of the total amount of tritium in the venous blood samples.
Perfusion 1: 90 nmoles of 17α,20α-dihydroxy-4-pregnen-3-one (17,20-P) were infused over a 20 min period after perfusion for $2\frac{1}{2}$ h.
Perfusion 2: as 1, in this case 4-^{14}C-labelled inhibitor has been used.
Perfusion 3: 90 nmoles of the inhibitor 17,20-P were infused over a two hour period, starting at $2\frac{1}{2}$ h.

Fig. 10. 4-^{14}C-17α,20α-Dihydroxy-4-pregnen-3-one, isolated from the testicular venous blood of perfusion 2 (fig. 9). The values are expressed as percentage of the total amount of ^{14}C-inhibitor which was infused and these values were not corrected for losses incurred to the isolated procedure. At the end of the experiment, 0.6% of the ^{14}C-inhibitor could be isolated from the testis.

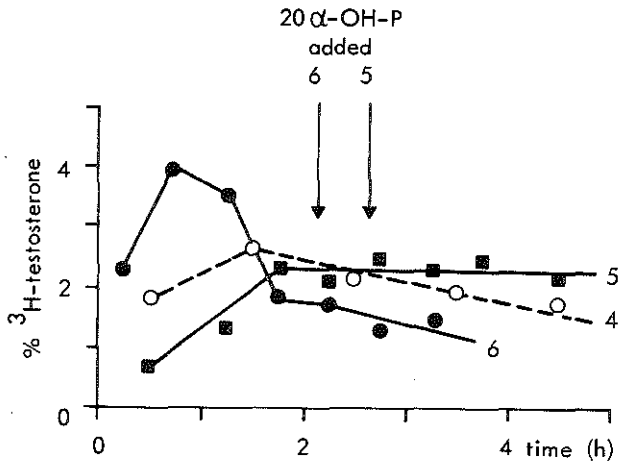


Fig. 11. ^{3}H-Incorporation into testosterone, isolated from the testicular venous blood after in vitro perfusion of rabbit testis with 17α-^{3}H-progesterone.
^{3}H-Testosterone values are given as percentage of the total amount of tritium in the venous blood samples.
Perfusion 4: control experiment, no inhibitor was infused.
Perfusion 5 and 6: 65 nmoles of 20α-dihydroprogesterone (20α-OH-P) were infused over a 20 min period, starting at 2½ h and 2 h respectively (indicated by arrow).

Perfusion experiments using the 20α-dihydroprogesterone, a better inhibitor in incubation experiments, also had no effect on the ^{3}H-testosterone secretion (fig. 11). From the perfusate collected at the time of inhibitor infusion, sufficient 20α-dihydroprogesterone could be isolated to cause an ultra-violet light absorbing zone on the paper strip after Bush AII paper chromatography, an indication for at least a 30-40 nmoles of steroid. Thus, absence of inhibition cannot be explained by metabolism of the inhibitors.

The testosterone secretion, estimated in the same experiments, did not change during the infusion of inhibitors.

1.4.4 Inhibition of $C_{17,20}$-lyase by 17β-ureido-1,4-androstadien-3-one

17β-Ureido-1,4-androstadien-3-one was found to be a competitive inhibitor with a high affinity for the rat testis $C_{17,20}$-lyase (apparent K_i-value in the range of 0.1-0.3 μM, fig. 12) From fig. 13 it is not clear if this steroid inhibited the conversion of labelled 17α-hydroxyprogesterone to testosterone, when it was infused during rabbit testis perfusions (final concentration in the perfusion medium 2-3 μM).

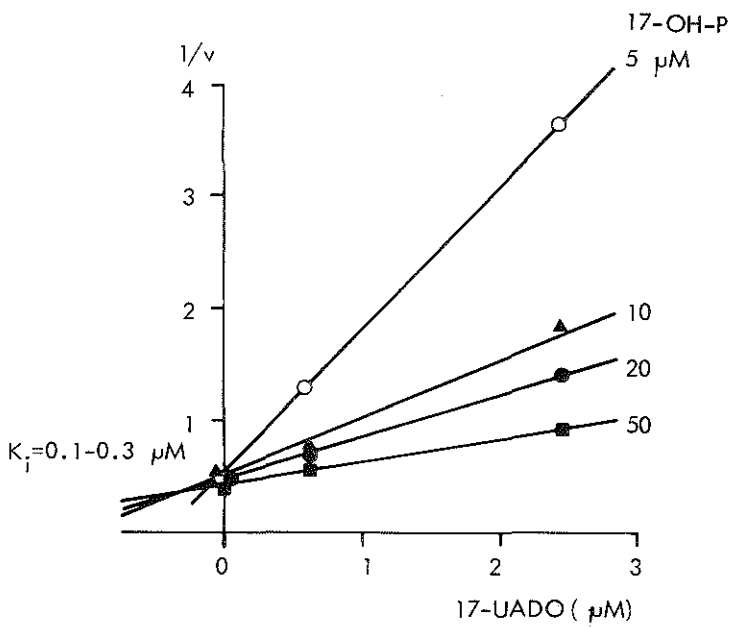


Fig. 12. Inhibition of $C_{17,20}$-lyase activity by 17 β-ureido-1,4-androstadien-3-one (17-UADO) in a washed 900 - 105,000 x g fraction of rat testis (1.3 mg of protein per incubation), represented in a Dixon type plot[97].
17 α-Hydroxyprogesterone (17-OH-P) was used as a substrate. The velocity is given as nmoles of androstenedione and testosterone formed per mg of protein per h.

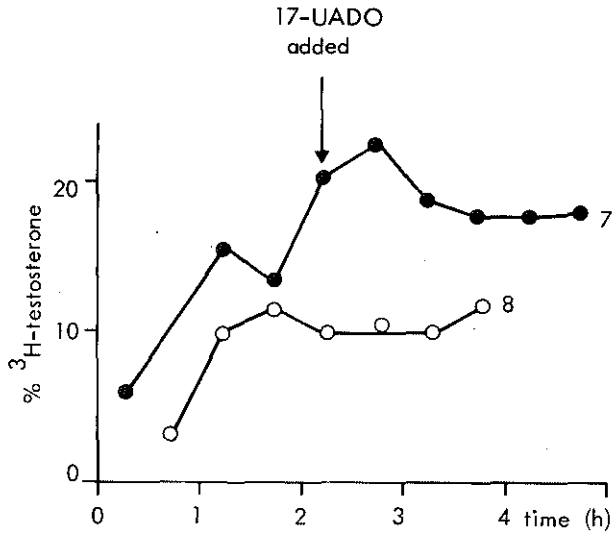


Fig. 13. ^{3}H-Testosterone, isolated from the testicular venous effluent after in vitro perfusion of rabbit testis with 7α-^{3}H-progesterone. The testis was perfused with rabbit erythrocytes (30%, by vol.), suspended in Krebs-Ringer bicarbonate buffer (pH 7.4), containing 4% of bovine serum albumine. ^{3}H-Testosterone values are given as percentage of the total amount of tritium in the venous effluent samples.
Perfusion 7 and 8: 20 nmoles of 17β-ureido-1,4-androstadien-3-one (17-UADO) were infused over a 20 min period, starting at 2 h as indicated by the arrow.

Goldman demonstrated that administration of steroid isoxazole, an inhibitor with a high affinity for the 3β-hydroxysteroid dehydrogenase, leads to accumulation of this inhibitor in the adrenal and ovary of female rats[77]. The higher incorporation of progesterone label into testosterone in this perfusion experiment (fig. 13), when compared with previous experiments (fig. 9 and 11), was probably caused by the absence of "corticosteroid binding globulin" in the perfusion medium.

Table 11 COMPARISON OF THE INHIBITION OF $C_{17,20}$-LYASE ACTIVITY BY 17β-UREIDO-1,4-ANDROSTADIEN-3-ONE (17-UADO) IN RAT AND RABBIT TESTIS.

7α-^{3}H-17α-Hydroxyprogesterone (5 μM) was incubated with the 900-105,000xg fractions of rat and rabbit testis (1.3 and 2.1 mg of protein respectively). Conversions are given as the total percentage of ^{3}H in the $C_{17,20}$-lyase products. The control rates of conversion were 1.4 and 0.9 nmoles per mg of protein per 30 min

Exp.	Species	% ^{3}H in $C_{17,20}$-lyase products			% inhibition
		control	17-UADO 0.6 μM	17-UADO 9.0 μM	
1	rat	47	18	-	61
		49	21	-	
2	rabbit	31	-	24	23
		30	-	22	

A comparison between the rat and rabbit testis revealed that a much higher concentration of the 17β-ureido-1,4-androstadien-3-one was necessary for inhibition of the rabbit testis lyase (table 11). This less efficient inhibition can be explained by species differences in the properties of the $C_{17,20}$-lyase but might also be indicative of the presence of two lyases, one specific for 17α-hydroxyprogesterone and one for the 17α-hydroxypregnenolone (the latter being found predominantly in rabbit testis).

It is therefore desirable that the screening procedure for specific inhibitors of human testosterone production should include various animal species.

1.4.5 Conclusions and discussion

It is concluded that during in vitro incubation 17α, 20α-dihydroxy-4-pregnen-3-one and 20α-dihydroprogesterone can inhibit the $C_{17,20}$-lyase activity of both rat and rabbit testis. 20α-Dihydroprogesterone is a competitive inhibitor of the rat testis enzyme (apparent K_i = 2-5 μM). These potential inhibitors, added to the arterial blood in concentrations of 10 μM, do not inhibit the $C_{17,20}$-lyase in perfused rabbit testis.

The presence of albumin can partially mask the inhibitory effects. Therefore in vitro incubations with dilute subcellular fractions in the absence of albumin may lead to a high degree of inhibition. On the other hand, in total tissue extracts or in the intact cell inhibitions might be less effective because of the presence of relatively high concentrations of cytosolic proteins.

The absence of inhibition during the perfusion experiments might also result from a poor penetration of 20α-dihydroprogesterone and 17α,20α-dihydroxyprogesterone into the tissue. However the following arguments make this explanation difficult to accept:

a. 20α-dihydroprogesterone apparently did enter rapidly into minced rat testis tissue during incubation experiments (fig. 7).
b. the concentration of inhibitor in the perfusion medium (approximately 10 μM) was much larger than the progesterone concentration (1-3 nM), while only an inhibitor concentration three times as high as the substrate concentration was necessary for inhibition during incubation experiments with homogenate fractions.
c. Also after perfusion of both ^{3}H-progesterone and ^{14}C-17α,20α-dihydroxy-4-pregnen-3-one (fig. 9), both isotopes were present in the inhibitor isolated from the venous blood and testis tissue.

Calculations, according to Gurpide[117], of the uptake of inhibitor by the tissue unfortunately cannot be applied because this requires a continuous infusion of labelled inhibitor and steady state conditions. Therefore the amount of 17α,20α-dihydroxy-4-pregnen-3-one which had penetrated into the tissue remained unknown. Finally it must be kept in mind, that in the in vivo situation the 17α,20α-dihydroxy-4-pregnen-3-one is synthesized at the subcellular level, whereas in the perfusion experiments this compound was added to the arterial blood.

From the results of these perfusion experiments it cannot be concluded if 17α,20α-dihydroxy-4-pregnen-3-one is of importance for the regulation of testosterone biosynthesis in vivo.

1.5 Estimation of 17α,20α-dihydroxy-4-pregnen-3-one in testis tissue

1.5.1 Introduction

The evaluation of the role of 17α,20α-dihydroxy-4-pregnen-3-one as inhibitor of $C_{17,20}$-lyase made it necessary to develop a method for its estimation and determination in testis tissue. A comparison of the levels of 17α,20α-dihydroxy-4-pregnen-3-one with those of 17α-hydroxyprogesterone in testis tissue should reveal if enough inhibitor is present to compete with the substrate for the $C_{17,20}$-lyase.

The estimation method has been described in detail in paragraph 1.2.8. Table 12 gives the mean recoveries of 17α,20α-dihydroxy-4-pregnen-3-one at several stages of the estimation.

Table 12 RECOVERIES DURING ESTIMATION OF 300 PMOLES 17α,20α-DIHYDROXY-4-PREGNEN-3-ONE

The recovery is expressed as per cent tritium of the added internal standard. The mean of ten estimations and S.D. are given.

Mean recovery after	% tritium
1. Periodic acid oxidation and t.l.c.	58 ± 13
2. $NaBH_4$-reduction of androstenedione and t.l.c.	39 ± 11
3. Chloroacetylation of testosterone and t.l.c.	20 ± 8

Table 13 ACCURACY AND PRECISION OF THE ESTIMATION OF 17α,20α-DIHYDROXY-4-PREGNEN-3-ONE

Added (pmoles)	Estimated (pmoles)	S.D. (pmoles)	S.D. (%)	n
300	322	51	16	12
75	90	17	19	6

The accuracy and reproducibility of the method were calculated from a series of determinations of known amounts of 17α,20α-dihydroxy-4-pregnen-3-one added to a 0.9% NaCl solution, containing 1% of bovine serum albumin (table 13).

Estimations of "blank samples" without steroid gave results in the order of 1 to 5 pmoles of testosterone chloroacetate. After corrections for losses that occurred during the isolation procedure, an average blank value in the order of 15 pmoles was obtained. All the values given for 17α,20α-dihydroxy-4-pregnen-3-one, have been corrected for the mean blank value of the particular series of determinations. The sensitivity of the method is approximately 15 pmoles.

1.5.2 Concentrations of 17α-20α-dihydroxy-4-pregnen-3-one

The concentrations were estimated in testes of six rats and compared with the 17α-hydroxyprogesterone concentrations as measured by a competitive protein binding assay (see paragraph 1.2.7). The amounts of 17α,20α-dihydroxy-4-pregnen-3-one in testis tissue were small and often below the limits of detection.

Table 14 CONCENTRATIONS OF 17α-HYDROXYPROGESTERONE (17OH-P) AND 17α,20α-DIHYDROXY-4-PREGNEN-3-ONE (17,20-P) IN RAT TESTIS

The testes of each rat were assayed together (2.5-3 g wet weight), the values for 17OH-P are the mean of triplicate estimations.

rat	17OH-P (pmoles/g)	17,20-P (pmoles/g)
1	7	<5
2	24	<5
3	55	10
4	88	<5
5	100	20
6	33	13
mean ± S.D.	51 ± 38	(10)

It is evident from the results in table 14, however, that the levels of this potential inhibitor are at least a five times lower than those of 17α-hydroxyprogesterone. The concentrations of this steroid in rabbit testis (6-7 g each pair) were equally low and almost undetectable. It was not possible to detect significant amounts of 17α,20α-dihydroxy-4-pregnen-3-one in 20 minutes samples of testicular venous blood of rats and rabbits.

1.5.3 Discussion and conclusions

The specificity of the estimation of 17α,20α-dihydroxy-4-pregnen-3-one is guaranteed by the combination of the chemical conversions with chromatographic purifications. Androstenedione and testosterone, both present in the sam-

ples, were separated from the 17α,20α-dihydroxy-4-pregnen-3-one at the first chromatographic step. When ^{14}C-labelled testosterone was added to the samples, no ^{14}C-radioactivity was detectable in the purified fractions. Pregnane metabolites with a 17β,20-dihydroxy structure, if present, might have been converted to 5α(β)-androstane-3,17-dione[118] and subsequently to 17β-hydroxy-5α(β)-androstane-3-one chloroacetate, but the latter compound separates from testosterone chloroacetate during gas chromatography on a QF-1 column. The 20β-isomer of the inhibitor has a higher R_f-value after paper chromatography than the 20α-isomer. Moreover, the 20β-hydroxysteroid dehydrogenase activity is relatively low[119] and it is unlikely that the 20β-isomer can interfere.

The low recovery obtained after the isolation and purification procedure is compensated by the high sensitivity of the electron capture detection. A possibility for a more practical estimation method might be the gas chromatography of 17α,20α-dihydroxy-4-pregnen-3-one as the trimethylsilyl-ether. This latter possibility has been applied to the estimation of pregnanetriols[120]. This approach for our estimation might have resulted in higher recoveries, but the lower sensitivity of flame-ionization detection makes it doubtful whether such a method would be more sensitive.

The amounts of 17α,20α-dihydroxy-4-pregnen-3-one in rat testis tissue are low and only two or three times higher than the blank values. Moreover, one might expect that the method will lack specificity at these low levels and therefore the actual levels may be even lower than the estimated levels. Hence, it is concluded that the presence of endogenous 17α,20α-dihydroxy-4-pregnen-3-one in testis tissue is not certain and that, if present, its level is at least five times lower than the level of 17α-hydroxyprogesterone. Further consequences are discussed in paragraph 1.7.1.

1.6 Localization of 20α-hydroxysteroid dehydrogenase in rat testis

1.6.1 Introduction

Testosterone is produced in the Leydig cells of the interstitial tissue.[99] For the evaluation of the role of 17α,20α-dihydroxy-4-pregnen-3-one it is relevant to know if the 20α-hydroxysteroid dehydrogenase is preferentially localized in interstitial tissue or seminiferous tubules.

Fevold and Eik-Nes[121] determined the levels of several enzymes in the testis tissue from Passer domesticus in different stages of their annual testicular cycle. The relatively low 20α-hydroxysteroid dehydrogenase activity in testis with regressed spermatogenesis suggested that this enzyme was located in the germinal epithelium. Other observations indicate, however, that the situation in rat testis is more complex. The specific activity of the 20α-hydroxysteroid dehydrogenase in immature rat testis is comparable to the activity in adult rats[100]. Testes subjected to X-ray treatment[122] or experimental cryptorchidism[123] showed high activities and exposure of rats to a hot environment also resulted in a small increase in the 17α,20α-dihydroxy-4-pregnen-3-one formation in vitro[124,125]. These treatments result in a destruction of the germinal cells and therefore localization of the enzyme in the germinal cells is not likely. However, the Sertoli cells and the interstitium, which are not affected by these treatments, may be considered to contain 20α-hydroxysteroid dehydrogenase. The enzyme activity in rat testis was not increased by HCG treatment in vivo[126,127]. Baillie[128] cited unpublished work concerning the histochemical detection of 20α-hydroxysteroid dehydrogenase activity in the human Leydig cells.

Following the demonstration that interstitial tissue and seminiferous tubules from rat testis can be separated with a simple dissection technique, it is now possible to study directly the steroid biosynthesis and metabolism in these isolated structures[129,130]. Lacy et al.[131,132] described the formation of 20α-dihydroprogesterone from progesterone and pregnenolone by isolated seminiferous tubules of rat testis in vitro, although comparison on a quantitative basis with the interstitial tissue was not made.

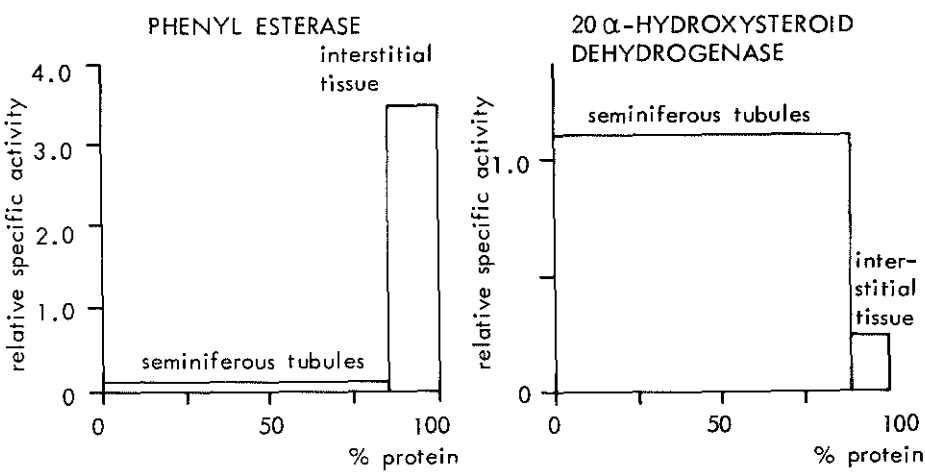


Fig. 14. Localization of 20α-hydroxysteroid dehydrogenase in rat testis. Phenyl esterase activity was determined in sonicated samples of total homogenates of the tissues, using p-nitrophenylacetate as a substrate. The 20α-hydroxysteroid dehydrogenase activity was estimated in the 105,000 x g supernatants with 17α-hydroxyprogesterone (30 μM) as a substrate, the specific activities in seminiferous tubules and interstitial tissue were respectively 39 and 8.4 pmoles per mg of protein per minute.

1.6.2 Results and discussion

The activities of the 20α-hydroxysteroid dehydrogenase were estimated in the soluble fractions from dissected seminiferous tubules and interstitial tissue from rat testis, using 17α-hydroxyprogesterone as a substrate (fig. 14). A non-specific phenyl esterase, localized in the interstitial tissue was used as an enzyme marker[95]. The separation factor between tubules and interstitium was 50 or better.

From the results in fig. 14 it is evident that the 20α-hydroxysteroid dehydrogenase is mainly localized in the seminiferous tubules. Some conversion can occur in the interstitium and it cannot be excluded that this enzyme activity reflected contamination with tubular material, because a marker enzyme for seminiferous tubules was not determined. Calculation of enzyme activity per gram of total testis shows that 97% of the enzyme is of tubular origin. Hence, if sufficient substrate is available, most of the 17α,20α-dihydroxy-4-pregnen-3-one will be produced in the tubules, and not at the site of testosterone formation in the Leydig cells. The 20α-hydroxysteroid dehydrogenase activity does not correlate with the development of the germinal cells[100,122-125] and therefore a localization in the Sertoli cells may be postulated.

1.7 Conclusions and general discussion

1.7.1 Conclusions

It appears from the results in paragraph 1.4, that the presence of endogenous 17α,20α-dihydroxy-4-pregnen-3-one in testis tissue from rats and rabbits is doubtful. In rat testis the levels of 17α,20α-dihydroxy-4-pregnen-3-one are at least five times lower than levels of 17α-hydroxyprogesterone. These low tissue levels render the possibilities for inhibition unfavourable, because it is known from the enzyme kinetic studies[1], that the apparent K_m-value of the $C_{17,20}$-lyase for 17α-hydroxyprogesterone is 2 μM, whereas the apparent K_i-value for 17α,20α-dihydroxy-4-pregnen-3-one is 9 μM. This reflects that the enzyme has a greater affinity for the substrate than for the inhibitor. Thus, as found in the incubation experiments (see paragraph 1.4.2), inhibitor concentrations higher than the substrate concentrations are required for effective inhibition. The results of estimations of 17α,20α-dihydroxy-4-pregnen-3-one indicate, however, that the opposite situation is actually found in the rat testis. Concentrations of 17α,20α-dihydroxy-4-pregnen-3-one are lower than 0.01 μM, whereas the concentration of 17α-hydroxyprogesterone is in the order of 0.05 μM. Moreover, 97% of the 20α-hydroxysteroid dehydrogenase activity of the rat testis is localized in the seminiferous tubules and the endogenous 17α,20α-dihydroxy-4-pregnen-3-one, if any, may not be produced at the site of the testosterone production.

It is concluded therefore that the 17α,20α-dihydroxy-4-pregnen-3-one has no function in the regulation of the $C_{17,20}$-lyase activity and testosterone production in the Leydig cells <u>in vivo</u>.

1.7.2 Subcellular distribution of steroids, binding to the endoplasmatic reticulum

Matsumoto and Samuels[49,134,135] found that, during incubation of progesterone and 17α-hydroxyprogesterone with a microsomal preparation of mouse testis, the incorporation of the progesterone label into androstenedione and testosterone was approximately two times higher than the incorporation of the 17α-hydroxyprogesterone label. When they tried to explain this result they observed that progesterone binds better to the microsomal particle than 17α-hydroxyprogesterone and this difference in binding could account for the isotope ratio in the products. Similar results have been obtained for rat and rabbit testes[136]. Burstein and Gut[137] interpreted these results by proposing a specific spatial arrangement of the enzymes on the microsomal surface, which can cause a direct transfer of the newly formed 17α-hydroxyprogesterone to the $C_{17,20}$-lyase without release of this intermediate into the medium. As a result, a free exchange between added and endogenously formed 17α-hydroxyprogesterone is hampered. If this theory is correct another argument is obtained for the inability of 17α,20α-dihydroxy-4-pregnen-3-one (either added exogenously or transported endogenously from the seminiferous tubules) to compete with $C_{17,20}$-lyase for the locally formed 17α-hydroxyprogesterone.

When interpreting the mean tissue concentration (see paragraph 1.7.1) an ever recurring point for discussion is the possibility for a locally high substrate concentration on or in the subcellular organella. From the foregoing discussion, it is clear that the formed 17α-hydroxyprogesterone, rather than the 17α,20α-dihydroxy-4-pregnen-3-one, is much more likely to concentrate on the endoplasmatic reticulum. This may support the conclusion from paragraph 1.7.1.

1.7.3 Localization of 20α-hydroxysteroid dehydrogenase: further consequences

^{3}H-Labelled 17α,20α-dihydroxy-4-pregnen-3-one was secreted during the perfusion experiments with ^{3}H-progesterone (see paragraph 1.3.2), but the rabbit testis *in situ* did not secrete detectable amounts of endogenously formed 17α,20α-dihydroxy-4-pregnen-3-one (see paragraph 1.5.2). This apparent discrepancy may be explained by the access of the substrate to the lyase and by the location of 20α-hydroxysteroid dehydrogenase activity. One can assume that during the perfusion experiments ^{3}H-progesterone also penetrates the seminiferous tubules, as has been demonstrated for several steroids[138-141]. In addition these tubules also contain 17α-hydroxylase activity[129,130], so that ^{3}H-17α,20α-dihydroxy-4-pregnen-3-one can be formed and secreted. The cholesterol side-chain cleaving enzyme is not active in the seminiferous tubules[130,142] and *in vivo* the essential substrates will primarily be available in the Leydig cells. Apparently the seminiferous tubules either do not utilize 17α-hydroxyprogesterone from the Leydig cells for 20α-reduction or the product is rapidly metabolized. The testicular 3α-hydroxysteroid dehydrogenase and 5α-reductase activities may be preferentially located in the seminiferous tubules[108,132] and at least this 5α-reductase can also reduce C_{21}-steroids[143]. Therefore, accumulation of 17α,20α-dihydroxy-4-pregnen-3-one may also be prevented by further metabolism.

1.7.4 The possible function of 20α-hydroxysteroid dehydrogenase

A possible function of the 20α-hydroxysteroid dehydrogenase in testis may be the elimination of 17α-hydroxyprogesterone as a precursor for testosterone rather than the production of an inhibitor for the $C_{17,20}$-lyase. A similar function has been proposed for the testicular 5α-reductase[103,106]. Increasing ovarian 20α-hydroxysteroid dehydrogenase activity is a sensitive index for luteolysis in rat and rabbit ovaries and causes a fall in progesterone secretion and a concomittant rise in the 20α-dihydroprogesterone secretion[145]. The cessation of progesterone secretion by the rat ovary, which precedes parturation and lactogenesis at the end of the pregnancy, is primarily the consequence of the sharp increase in 20α-hydroxysteroid dehydrogenase activity[146,147]. Both examples illustrate the inactivation of progesterone by conversion to a metabolite with hardly any progestational activity. A possible function of 17α-hydroxyprogesterone metabolism in the seminiferous tubules of testis, however, is unknown.

1.8 References

1 Inano,H., Nakano,H., Shikita,M. and Tamaoki,B.(1967) Biochim.Biophys.Acta 137,540-548

2 Shikita,M., Inano,H. and Tamaoki,B.(1967)Biochemistry 6, 1760-1764

3 Inano,H. and Tamaoki,B.(1971)Biochim.Biophys.Acta 239,482-493

4 Notation,A.D. and Ungar,F.(1968)Can.J.Biochem. 46,1185-1190

5 Notation,A.D. and Ungar,F.(1969)Steroids 14,151-159

6 Notation,A.D. and Ungar,F.(1969)Biochemistry 8,501-506

7 Payne,A.H., Mason,M. and Jaffe,R.B.(1969)Steroids 14,685-704

8 Payne,A.H.(1972)Biochim.Biophys.Acta 258,473-483

9 Wiener,M. and Allen,S.H.G.(1967)Steroids 9,567-582

10 Wiener,M. and Allen,S.H.G.(1968)Steroids 12,261-273

11 Jensen,V., Carson,P. and Deshpande,N.(1971)J.Endocrinol. 50, 177-178

12 Jensen,V., Carson,P. and Deshpande,N.(1972)J.Endocrinol. 55, 311-321

13 Neher,R. and Kahnt,F.W.(1965)Experientia 21,310-312

14 Kaartinen,E., Laukkanen,M. and Saure,A.(1971)Acta Endocrinol. 66,50-64

15 Burrow,G.N., Mulrow,P.J. and Bondy,P.K.(1966)Endocrinology 79, 955-963

16 Morrow,L.B., Burrow,G.N. and Mulrow,P.J.(1967)Endocrinology 80,883-888

17 Burrow,G.N.(1969)Endocrinology 84,979-985

18 Weinstein,R.L. and Kliman,B.(1972)Metabolism 21,99-106

19 Sauer,L.A.(1972)Arch.Biochem.Biophys. 149,42-51

20 Axelrod,L.R.(1965)Biochim.Biophys.Acta 97,551

21 Yanaihara,T. and Troen,P.(1972)J.Clin.Endocrinol.Metab. 34,783

22 Arth,G.E., Patchett,A.A., Jefopoulos,T., Bugianesi,R.L., Peterson,L.H., Hamm,E.A., Kuehl,F.A. and Brink,N.G.(1971) J.Med.Chem. 14,675-681

23 Haksar,A., Romanoff,E.B., Hagino,N. and Pincus,G.(1967) Steroids 9,405-414

24 Haksar,A. and Romanoff,E.B.(1968)Steroids 12,345-357

25 Cook,B., Niswender,G.D., Sutterlin,N.S., Norton,H.W. and Nalbandov,A.V.(1968)Steroids 11,321-336
26 Hall,P.F.(1970)in "The Testis"(Johnson,A.D., Gomes,W.R. and VanDemark,N.L.,eds),Vol.2, p.10,Academic Press,New York
27 Dexter,R.N., Fishman,L.M. and Ney,R.L.(1970)Endocrinology 87, 836-846
28 Ichii,S., Forchielli,E. and Dorfman,R.I.(1963)Steroids 2, 631-656
29 Dorfman,R.F., Sharma,D., Menon,K.M.J. and Forchielli,E.(1967) Proc. Third Asia and Oceania Congr. Endocrinology, pp.171-177, Manilla
30 Koritz,S.B. and Hall,P.F.(1964)Biochemistry 3,1298-1304
31 Koritz,S.B. and Kumar,A.M.(1970)J.Biol.Chem. 245,152-159
32 Farese,R.V.(1971)Endocrinology 89,958-962
33 Simpson,E.R., Jeffcoate,C.R., Brownie,A.C. and Boyd,G.S.(1972) Eur.J.Biochem. 28,442-450
34 Menon,K.M.J., Drosdowsky,M., Dorfman,R.I. and Forchielli,E. (1965)Steroids,Suppl.I,95-111
35 Colby,H.D. and Kitay,J.I.(1972)Steroids 20,143-151
36 Brownie,A.C., Colby,H.D., Gallant,S. and Skelton,R.S.(1970) Endocrinology 86,1085-1092
37 Péron,F.G., Moncloa,F. and Dorfman,R.I.(1960)Endocrinology 67, 379-388
38 Birmingham,M.K. and Kurlents,E.(1958)Endocrinology 62,47-60
39 Jensen,P.K.(1959)Nature(London) 184,451-452
40 Stoppani,A.O.M., de Brignone,C.M.C. and Brignone,J.A.(1968) Arch.Biochem.Biophys. 127,463-475
41 Sharma,D.C., Forchielli,E. and Dorfman,R.I.(1963)J.Biol.Chem. 238,572-575
42 Colby,H.D. and Brownie,A.C.(1971)Biochem.Pharmacol. 20,803-813
43 Brownie,A.C., Colby,H.D. and Simpson,E.R.(1971)in Proc. Third Int. Congr. Hormonal Steroids(James,V.H.T. and Martini,L.eds) pp.285-289,Excerpta Medica Foundation,Amsterdam
44 Kitay,J.F.(1968)in "Functions of the Adrenal Cortex" (McKerns,K.W.)Vol.2, p.786,North Holland Publishing Company, Amsterdam
45 Sharma,D.C. and Dorfman,R.I.(1964)Biochemistry 3,1093-1097

46 Nayfeh,S.N. and Baggett,B.(1969)Steroids 14,269-283
47 Huseby,R.A., Dominguez,O.V. and Samuels,L.T.(1961) Rec.Progr.Hormone Res. 17,1-42
48 Mahajan,D.K. and Samuels,L.T.(1962)Fed.Proc. 21,209
49 Samuels,L.T., Matusumoto,K., Aoshima,Y. and Bedrak,E.(1969) in "Progress in Endocrinology"(Gual,C. ed),pp.845-850, Excerpta Medica Foundation,Amsterdam
50 Deshpande,N., Jensen,V., Carson,P., Bulbrook,R.D. and Doouss,T.W.(1970)J.Endocrinol. 47,231-242
51 Eik-Nes,K.B.(1970)in "The Androgens of the Testis" (Eik-Nes,K.B.,ed),p.22,Marcel Dekker,New York
52 Goldman,A.S.(1967)J.Clin.Endocrinol.Metab. 27,320-324
53 Patwardhan,V.V. and Lanthier,A.(1970)in Abstr. Third Int. Congr. Hormonal Steroids, Int. Congr. Series 210, p.110, Excerpta Medica Foundation,Amsterdam
54 Kowal,J., Forchielli, E. and Dorfman,R.I.(1964)Steroids 4, 77-100
55 Yanaihara,T., Troen,P., Troen,B.R. and Troen,M.L.(1972) J.Clin.Endocrinol.Metab. 34,968-973
56 Krüskemper,H.L., Forchielli,E. and Ringold,H.J.(1964) Steroids 3,295-309
57 Ewald,W., Werbin,H. and Chaikoff,I.L.(1964)Biochim.Biophys. Acta 81,199
58 Ewald,W. and Weber,K.M.(1969)Acta Endocrinol.Suppl. 138,51
59 Milgrom,E. and Beaulieu, E.E.(1970)Steroids 15,563-588
60 Mousseron-Canet,M., Borgna,J.L., Beziat,Y. and Chavis,C. (1968)C.R.Acad.Sci.Paris 267,797-800
61 Purdy,R.H., Malla,M. and Little,B.(1964)Biochim.Biophys.Acta 89,557-560
62 Ruokonen,A., Laatikainen,T., Laitinen,E.A. and Vihko,R. (1972)Biochemistry 11,1411-1416
63 Garren,L.D., Hill,G.N., Masui,H. and Walton,G.M.(1971) Rec.Progr.Hormone Res. 27,433-474
64 Koritz,S.B., Yun,J. and Ferguson,J.J.(1968)Endocrinology 82, 620-622
65 McCune,R.W., Roberts,S. and Young,P.L.(1970)J.Biol.Chem. 245 3859-3867

66 Berger,G.M.B. and Jones,J.D.(1971)Endocrinology 89,722-729
67 Sulimovici,S. and Boyd,G.S.(1969)Eur.J.Biochem. 7,549-558
68 Sulimovici,S.(1970)in Abstr. Third Int. Congr. Hormonal Steroids, Int. Congr. Series 210, p.117,Excerpta Medica Foundation,Amsterdam
69 Kinson,G.A.(1971)Fed.Proc. 30,Abstract 1568
70 Ellis,L.C.(1972)Endocrinology 90,17-28
71 Neville,A.M. and Engel,L.L.(1968)J.Clin.Endocrinol.Metab. 28, 49-60
72 Goldman,A.S.(1968)J.Clin.Endocrinol.Metab. 28,1539-1546
73 Farese,R.V.(1970)Steroids 15,245-250
74 Goldman,A.S.(1970)Endocrinology 86,583-589
75 Goldman,A.S.(1969)Endocrinology 85,325-329
76 Bloch,E., Lew,M. and Klein,M.(1971)Endocrinology 89,16-31
77 Goldman,A.S.(1970)Endocrinology 86,678-686
78 Lipner,H. and Greep,R.O.(1971)Endocrinology 88,602-607
79 Gaunt,R., Steinetz,B.G. and Chart,J.J.(1968)Clin.Pharmacol. Ther. 9,657-681
80 Dorfman,R.I. and Ungar,F.(1965)in "Metabolism of Steroid Hormones", p.135 and p.181,Academic Press,New York
81 Tamaoki,B. and Shikita,M.(1966)in "Steroid Dynamics"(Pincus,G., Nakano,T. and Tait,J.F.,eds),pp.515-518,Academic Press,New York
82 Inano,H., Inano,A. and Tamaoki,B.(1970)J.Steroid Biochem. 1, 83-91
83 Goldman,A.S.(1971)Endocrinology 88,527-531
84 Bray,G.A.(1960)Anal.Biochem. 1,279
85 Neher,R.(1964)in "Steroid Chromatography",2nd edn,pp.96-97, Elsevier Publishing Co,Amsterdam
86 Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J.(1951) J.Biol.Chem. 193,265-275
87 Hall,P.F.(1965)Endocrinology 76,396
88 Levier,R. and Spaziani,E.(1969)J.Exp.Zool. 169,113
89 Brownie,A.C., van der Molen,H.J., Nishizawa,E.E. and Eik-Nes, K.B.(1964)J.Clin.Endocrinol.Metab. 24,1091-1102
90 de Jong,F.H. and van der Molen,H.J.(1970)Ann.Clinical Research 2,381-392
91 Nayfeh,S.N. and Bagett,B.(1966)Endocrinology 78,460-470

92 Shikita,M., Ogiso,T. and Tamaoki,B.(1969)Endocrinol.Japon. 16,11-16
93 Bardin,W.C. and Lipsett,M.B.(1967)Steroids 9,71-84
94 Christensen,A.K. and Mason,N.R.(1965)Endocrinology 76,646-656
95 Rommerts,F.F.G., van Doorn,L.G., Galjaard,H., Cooke,B.A. and van der Molen,H.J.(1973)J.Histochem.Cytochem.(accepted for publication)
96 Tamaoki,B. and Shikita,M.(1966)in "Steroid Dynamics" (Pincus,G., Nakao,T. and Tait,J.F.,eds),pp.493-528,Academic Press,New York
97 Dixon,M.(1953)Biochem.J. 55,170-171
98 Eik-Nes,K.B.(1970)in "The Androgens of the Testis" (Eik-Nes,K.B.,ed),pp.14-23,Marcel Dekker,New York
99 Hall,P.F.(1970)in "The Testis"(Johnson,A.D., Gomes,W.R. and VanDemark,N.L.,eds),Vol.2, pp.18-31,Academic Press,New York
100 Inano,H. and Tamaoki,B.(1966)Endocrinology 79,579-590
101 Nayfeh,S.N. and Baggett,B.(1966)Endocrinology 78,460-470
102 Gower,D.B.(1966)Steroids 8,511-526
103 Ficher,M. and Steinberger,E.(1971)Acta Endocrinol. 68,285-292
104 Steinberger,E. and Ficher,M.(1971)Endocrinology 89,679-684
105 Folman,Y., Sowell,J.G. and Eik-Nes,K.B.(1972)Endocrinology 91, 702-710
106 Coffey,J.C., French,F.S. and Nayfeh,S.N.(1971)Endocrinology 89,865-872
107 Rivarola,M.A., Podestã,E.J. and Chemes,H.E.(1972) Endocrinology 91,537-542
108 Rivarola,M.A.,and Podestã,E.J.(1972)Endocrinology 90,618-624
109 Payne,A.H. and Jaffe,R.B.(1970)Endocrinology 87,316-322
110 Strickland,A.L., Nayfeh,S.N. and French,F.S.(1970)Steroids 15,373-387
111 Shikita,M., Ogiso,T. and Tamaoki,B.(1969)Endocrinol.Japon. 16,11-16
112 Inano,H., Tsuno,K. and Tamaoki,B.(1970)Biochemistry 9, 2253-2259
113 Sandberg,A.A., Rosenthal,H., Schneider,S.L. and Slaunwhite, W.R.(1966)in "Steroid Dynamics"(Pincus,G., Nakao,T. and Tait,J.F.,eds),p.49,Academic Press,New York

114 Billiar,R.B. and Little,B.(1971)Endocrinology 88,263-266
115 Billiar,R.B., Tanaka,Y., Knappenberger,M., Hernandez,R. and Little,B.(1969)Endocrinology 84,1152-1160
116 Hamilton,M.A., McCune,R.W. and Roberts,S.(1972)J.Endocrinol. 54,297-315
117 Gurpide,E. and Welch,M.(1969)J.Biol.Chem. 244,5159-5169
118 Menini,E. and Norymberski,J.(1965)Biochem.J. 95,1-16
119 Inano,H. and Tamaoki,B.(1966)Endocrinology 79,579-590
120 van der Molen,H.J.(1968)in "Gas Phase Chromatography in Steroids"(Eik-Nes,K.B.,ed),p.218,Springer Verlag,Berlin
121 Fevold,H.R. and Eik-Nes,K.B.(1962)Gen.Comp.Endocrinol. 2, 506-515
122 Schoen,E.J.(1964)Endocrinology 75,56-65
123 Inano,I. and Tamaoki,B.(1968)Endocrinology 83,1074-1082
124 Bedrak,E., Samoiloff,V., Sod-Moria,U.A. and Goldberg,S. (1971).J.Endocrinol. 51,489-498
125 Bedrak,E.(1972)Gen.Comp.Endocrinol. 18,525-533
126 Schoen,E.J. and Samuels,L.T.(1965)Acta Endocrinol. 50,365-
127 Shikita,M. and Hall,P.F.(1967)Biochim.Biophys.Acta 136,484-497
128 Baillie,A.H., Ferguson,M.M. and Hart,D.McK.(1966)in "Development in Steroid Histochemistry",p.55,Academic Press, London
129 Christensen,A.K. and Mason,N.R.(1965)Endocrinology 76,646-656
130 Hall,P.F., Irby,D.C. and de Kretser,D.M.(1969)Endocrinology 84,488-496
131 Lacy,D. and Pettitt,A.J.(1970)Brit.Med.Bull. 26,87-91
132 Bell,J.B.G., Vinson,G.P. and Lacy,D.(1971)Proc.Roy.Soc. Lond.B 176,433-443
133 Tamaoki,B.(1973)J.Steroid Biochem. 4,89-118
134 Matsumoto,K. and Samuels,L.T.(1969)Endocrinology 85,402-409
135 Matsumoto,K., Monden,Y. and Samuels,L.T.(1972)in Abstract 22, Fourth Int. Congress of Endocrinology,Washington,Int. Congress Series 256,p.9,Excerpta Medica Foundation,Amsterdam
136 van der Molen,H.J. and de Bruijn,H.W.A.(1971)in "Gonadal Steroid Secretion"(Baird,E.,ed),pp.96-123,Edinburgh University Press,Edinburgh

137 Burstein,S. and Gut,M.(1971)Rec.Progr.Hormone Res. 27,324-326
138 Galjaard,H., van Gaasbeek,J.H., de Bruijn,H.W.A. and van der Molen,H.J.(1970)J.Endocrinol. 48,li-lii
139 Parvinen,M., Hurme,P. and Niemi,M.(1970)Endocrinology 87, 1082-1084
140 Parvinen,M. and Niemi,M.(1971)Steroidologica 2,129-137
141 van Doorn,L.G., de Bruijn,H.W.A., Galjaard,H. and van der Molen,H.J.(1973)Biol. of Reprod. (submitted for publication)
142 van der Vusse,G.J., Kalkman,M.L. and van der Molen,H.J.(1973) Biochim.Biophys.Acta 297,179-185
143 Oshima,H., Sarada,T., Ochiai,K. and Tamaoki,B.(1970) Endocrinology 86,1215-1224
144 Cooke,B.A., de Jong,F.H., van der Molen,H.J. and Rommerts, F.F.G.(1972)Nature New Biology 237,255-256
145 Strauss,J.F., Foley,B. and Stambaugh,R.(1972)Biol. of Reprod. 6,78-86
146 Kuhn,N.J. and Briley,M.S.(1970)Biochem.J. 117,193-201
147 Bast,J.D. and Melampy,R.M.(1972)Endocrinology 91,1499-1505

Part 2 The role of Δ^{5},3β-hydroxysteroids

2.1 Introduction

2.1.1 Alternative pathways for steroid biosynthesis

Steroid hormones can be formed along the Δ^5,3β-hydroxysteroid and the Δ^4,3-oxosteroid pathways (see GENERAL INTRODUCTION, fig. 1). Possibilities for the existence of other pathways are, however, still being investigated.

Δ^4,3-oxosteroid pathway

Recently two reports were published concerning possible alternative routes in the Δ^4,3-oxosteroid pathway. Nakano et al.[1] described that the micro-organism Cladosporicum resinae can transform progesterone directly to testosterone acetate, by-passing 17α-hydroxyprogesterone and androstenedione. The testosterone acetate is subsequently hydrolized by an esterase to testosterone. However, testosterone acetate could not be detected in rat testis and addition of an esterase inhibitor did not affect the rate of testosterone formation in vitro[2]. Several other potential possible pathways from progesterone to C_{17}-steroids have been explored but the possibility of a route by-passing 17α-hydroxyprogesterone could not be established[3,4]. Therefore, it is generally accepted that the route along 17α-hydroxyprogesterone and androstenedione is the predominant pathway from progesterone to testosterone in mammalian testis.

Δ^5,3β-hydroxysteroid pathway

Recent investigations on alternative pathways have been concerned with the formation of Δ^5,3β-hydroxysteroids and the role of steroid sulfates in hormone production. Theoretically, biosynthesis of C_{19}-steroids could occur through $C_{17,20}$-cleavage of cholesterol to dehydroepiandrosterone, excluding pregnenolone and 17α-hydroxypregnenolone as intermediates. Burstein and Dorfman[5] and Gual et al.[6] indicated this possibility in the discussion of their results of an investigation in an adrenal adenoma. Jungmann claimed the existence of this new pathway[7,8], when he believed to have isolated radioactivity in an eightcarbon fragment, the 6-methylheptan-2-one, after incubation of 26-^{14}C-cholesterol with homogenates of rat ovaries, testes and adrenal glands[7] and with acetone-dried powders of calf testes[8]. In a thorough reinvestigation of this problem Burstein et al.[9] and Hochberg et al.[10] could not obtain evidence for the formation of the C_8-fragment and it was concluded that Jungmann must have isolated an artefact. Shimuzu[11] reported the conversion of 17α,20α-dihydroxycholesterol to dehydroepiandrosterone by slices of normal human adrenal tissue, but again Burstein et al.[12] found no evidence for the formation of dehydroepiandrosterone from either cholesterol or its 17α,20α-dihydroxyderivates by acetone-dried powders of guinea pig adrenals. In a review on the biosynthesis of pregnenolone, Burstein and Gut[13] reiterated these conflicting observations and they presented additional evidence for the obligatory role of $C_{20,22}$-cleavage of cholesterol for steroid biosynthesis.

steroid sulfates

Cholesterol sulfate occurs in a variety of mammalian tissues and has been shown to be converted to several steroid sulfates in man *in vivo*[14]. Whether cholesterol sulfate only serves as a precursor of steroid sulfates, or whether it also participates in the biosynthetic pathways which lead to biologically active, unconjugated steroids is not entirely clear at present[13,14]. Moreover, a variety of tissues is capable of sulfo-conjugation of steroids *in vitro*[15]. The complexity of the role of steroid sulfates can be demonstrated by the findings of Telegdy *et al.*[16] on steroid biosynthesis in the foetus at midgestation. They investigated the incorporation of ^{3}H-cholesterol and ^{14}C-acetate into steroids and found that pregnenolone and dehydroepiandrosterone (free and conjugated) accounted for 95% of the steroid products. The $^{14}C/^{3}H$ ratio in these steroids, isolated from the conjugated fraction of the perfusates was much higher than that of the unconjugated compounds. They suggested therefore that in the foetus acetate is utilized predominantly via the conjugated pathway, whereas circulating cholesterol is mainly converted via the unconjugated pathway.

A number of sulfate conjugated steroids are present in high concentrations in human testis[17]. The normal human testis secretes the (mono)-sulfates of testosterone, pregnenolone and possibly androstenediol[18]. After administration of HCG a considerable increase in the secretion of the mono-sulfates of dehydroepiandrosterone, androstenediol and pregnenolone was observed but no increase in the secretion of testosterone sulfate was found[19]. *In vitro* incubation studies with human[20,21] and rat[22-26] testes preparations demonstrated that steroid sulfates can be hydrolyzed to precursors of testosterone. In human testis this sulfatase activity correlates with serum testosterone levels and the "Leydig cell activity index"[21]. Although it has been shown

that steroid sulfatase activity is present in rat testis, the latter is not controlled by gonadotrophins[27]. The presence of steroid sulfates in rat testis has not been demonstrated.

Apart from being synthesized in testis tissue, steroid sulfates may also be supplied by the arterial blood. In perfusion experiments with dog testis it was demonstrated that after infusion of radioactive dehydroepiandrosterone sulfate a small conversion to testosterone occurred[28]. In similar experiments with human testis hardly any labelled testosterone could be detected[29,30], but this may be explained by the relatively short perfusion period (several minutes), and dilution of the radioactive substrate with dehydroepiandrosterone sulfate in human plasma.

It is apparent that the relative importance of individual intermediates or biosynthetic pathways may be different in the various species and in different endocrine glands. Furthermore the type of tissue preparation and the technique used to study the pathways will be of importance in this respect.

2.1.2 Scope of this study

Van der Molen and Eik-Nes[31], who perfused dog testes with blood containing ^{3}H or ^{14}C labelled pregnenolone observed that the specific radioactivity of the dehydroepiandrosterone was much lower than that of 17α-hydroxypregnenolone, particularly during administration of HCG or 3',5'-cAMP. This suggests that only a small fraction (5-10%) of the dehydroepiandrosterone originated from 17α-hydroxypregnenolone and that precursors, other than pregnenolone, might be involved. Alternatively, incomplete mixing of the various labelled steroids with the tissue steroid pools might also explain their results.

These results raise the question whether or not the

synthesis via Δ^5,3β-hydroxysteroid sulfates contributes to the dehydroepiandrosterone formation or whether $C_{17,20}$-side-chain cleavage of cholesterol is possible under these conditions. In this respect it is noteworthy that the work of Burstein et al.[9,12,13] on the side-chain cleavage of cholesterol was almost solely carried out with acetone-dried mitochondrial preparations.

In the present study the role of Δ^5,3β-hydroxysteroids in testosterone production was investigated in detail. Testes from rabbits were used for this study because incubation experiments[32] and preliminary perfusion experiments suggested that the Δ^5,3β-hydroxysteroid pathway is involved in testosterone biosynthesis in this species, like in the human testis[33,34]. The following aspects were investigated:

1. The relative importance of the Δ^4,3-oxosteroid pathway and the Δ^5,3β-hydroxysteroid pathway for testosterone production. Special attention was given to the role of androstenediol as an intermediate.
2. The existence of precursors of dehydroepiandrosterone and androstenediol other than pregnenolone and 17α-hydroxypregnenolone. For this purpose rabbit testes were perfused <u>in vitro</u> with ^{3}H-pregnenolone and the relationship between the specific radioactivities of the secreted steroids was determined. During these perfusion experiments the presence of steady-state conditions were maintained. A specific and sensitive technique was applied for the determination of Δ^5,3β-hydroxysteroids, which had previously been developed for the determination of dehydroepiandrosterone[35].
3. The presence of steroid sulfates and sulfatase activity in rabbit testis, because specific radioactivities of the steroid intermediates only give an indirect estimate of alternative biosynthetic pathways.

2.2 Materials and methods

2.2.1 Methods for investigating pathways in steroid biosynthesis

Many different techniques have been used to study the relative importance of pathways for steroid hormone biosynthesis. Recent methods generally involve incubation or perfusion of endocrine tissue with two labelled precursors, with subsequent analysis of the labelled products. Occasionally the production of steroids from endogenous sources is measured in addition to the conversion of labelled precursor and this technique has been used in this study. Some of these methods are discussed below.

Experiments with two labelled precursors

Much information has been obtained from double label incubation experiments, with separately labelled ^{3}H and ^{14}C substrates. For example, when using ^{3}H-pregnenolone and ^{14}C-progesterone with steroid-forming tissues, the finding of a high $^{3}H/^{14}C$ ratio in androstenedione may indicate the dominance of the Δ^{5},3β-hydroxysteroid pathway. This has been observed for the normal human ovary[36,37] and a granulosa cell tumor[38]. The same principle was applied in incubation experiments with horse testis[39]. Incubation with ^{3}H-dehydroepiandrosterone and ^{14}C-17α-hydroxyprogesterone was carried out for determining the importance of androstenediol as intermediate in testosterone biosynthesis in human testis[33,40] and rabbit testis.

In order to obtain correct quantitative information on the relative importance of the various pathways, many analyses at various time intervals must be carried out and the

preferred pathway can then be derived from the sequential maxima in isotope incorporation of the different products[41,42]. From the results of incubations with ^{3}H-pregnenolone and ^{14}C-progesterone as substrates it was concluded that only the Δ^{4},3-oxosteroid pathway is of importance for the rat testis interstitial tissue[43] and that in human testis the Δ^{5},3β-hydroxysteroid pathway dominates[33]. In the same way it was demonstrated that transformation through 17α-hydroxypregnenolone was the major pathway for corticosteroid biosynthesis in an adrenal tumor[75].

Extrapolation of these results to the in vivo situation is difficult because the rate of penetration of added steroids into the tissue or differences in the binding of these substrates to the subcellular organella can influence the results[3,39,44]. The same disadvantages also hold for perfusion experiments which were performed with the human adrenal gland[45] and dog testis[46] with ^{3}H-dehydroepiandrosterone and ^{14}C-17α-hydroxyprogesterone.

A major disadvantage of all these experiments is, however, that no direct information is obtained on the steroid production from endogenous sources and that alternative biosynthetic pathways from other precursors might be overlooked. If tracer amounts of radioactive precursor are added to the incubation system, a homogeneous pool with the tissue endogenous precursors may not be formed[47-49]. Addition of large amounts of precursor (either radioactive or non-radioactive) decreases the relative contribution from endogenous sources and the biosynthetic pathway may then be biassed towards the contribution of the precursor; inhibition of the various steps of the pathway may also occur.

Steroid production and conversion of labelled precursor

One of the most important presumptions in the use of labelled compounds requires that the tissues endogenous

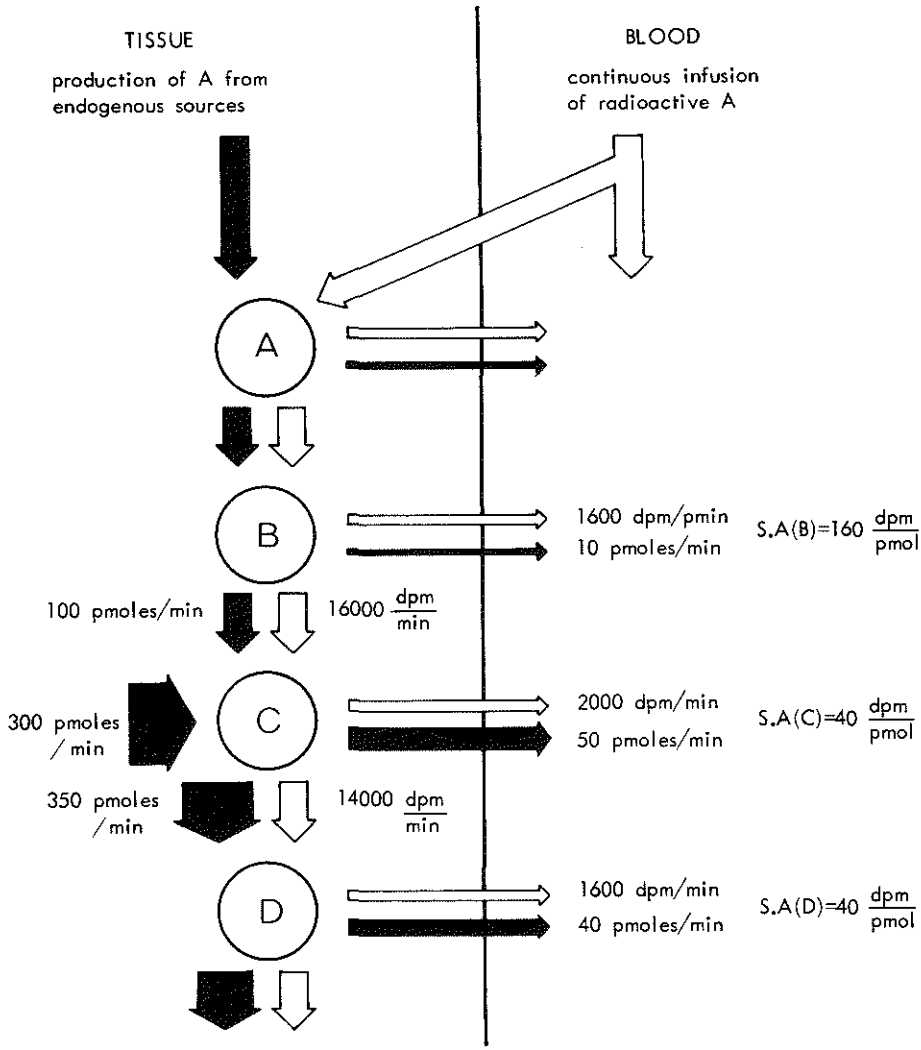


Fig. 15 Specific activities (S.A. dpm/pmol) of steroids A, B, C and D in a biosynthetic pathway when steady state conditions have been achieved during continuous infusion of radioactive steroid A. The open arrows represent the transfer rates or conversion rates of radioactive steroids (dpm/min), while the dark arrows represent these rates of the endogenous steroids (pmoles/min). The width of the arrows is proportional to the rate of transfer or of conversion. A second biosynthetic pathway to C, or a second, unlabelled tissue pool of C, causes a decreased specific activity of C, and subsequently of D.

precursors are metabolized along the same biosynthetic pathways as the added radioactive precursor. Zilversmit[50] showed on theoretical grounds that under certain steady state conditions the specific radioactivities of the products bore a definite relationship to one another. Assuming that steady state conditions are achieved through continuous perfusion with a trace amount of radioactive precursor A (fig. 15), it can be deduced that the specific radioactivity in the product B, C and D should be equal to each other and this is reflected by the specific activity in the fractions which are secreted. If, however, the specific activity in C is lower than that of B, another biosynthetic pathway or a second tissue pool contributes to unlabelled C[50,51]. The ratio R of the specific activities gives the fraction of the product originating from the known precursor, 1-R represents the fraction coming from unknown sources.

$$R = \frac{\text{specific radioactivity of product C}}{\text{specific radioactivity of precursor B}}$$

Pulse labelling technique

Steady state conditions are usually not achieved in normal incubation experiments or after a single injection of radioactive precursor in vivo. Nevertheless such experiments can, if correct mathematical analysis is applied, lead to an understanding of the behaviour of steroids towards the individual tissue compartments and steroid pools in terms of transfer rates, conversion rates and tissue concentrations[41,50,52]. In the interpretation of these data it may appear that simplified models of metabolic processes often contain so many unknown parameters that agreement with experimental data looses its significance. It is even possible to design different models which all equally satisfy the experimental results[53].

Constant infusion of radioactivity and the presence of a steady condition simplify the interpretation of the results and the estimation of unknown parameters, like transfer rates and conversion rates, becomes unnecessary.

Steady state conditions can also be achieved by incubation of tissue slices with frequent withdrawal of samples from the medium and addition of radioactive substrate or by applying the superfusion technique to tissue slices[42,54].

2.2.2 Materials

Some of the materials and basic techniques have already been described in paragraph 1.2.

16-^{3}H-Pregnenolone (S.A. 21 Ci/mM) was purchased from C.E.N., Mol (Belgium). The manufacturer specified that more than 98% of the tritium was attached to carbon 16, although its steric location was not known. 4-^{14}C-Pregnenolone (S.A. 55.7 mCi/mM) was obtained from Radiochemical Centre, Amersham and the ammonium salt of 4-^{14}C-dehydroepiandrosterone sulfate (58.8 mCi/mM) from New England Nuclear Corporation (Boston).

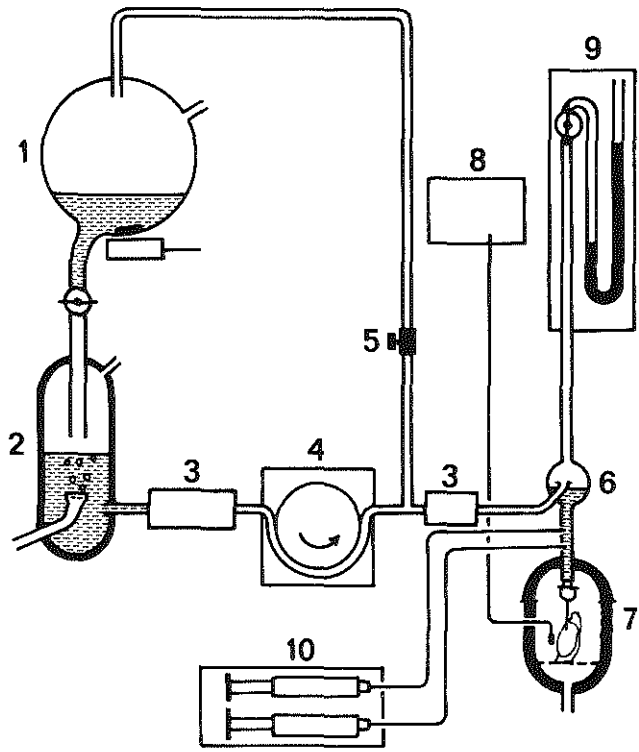
Androstenetrione was prepared as described by De Jong and Van der Molen[35] and was recrystallized successively from 1. methanol-water, 2. acetone-water and 3. cyclohexane-ethylacetate. Yellow crystals were obtained (m.p. 214-217^{o}C). This steroid is now commercially available (Steraloids Inc.). Pregnenetrione was obtained from Steraloids Inc.. HCG (Pregnyl, 3500 I.U./mg) and heparin were obtained from Organon (Oss).

2.2.3 Perfusion of rabbit testis in vitro

Rabbits of the New Zealand White strain, weighing 2.5-3.5 kg were used. The mean weight of a testis was 3.5 ± 0.6 g (S.D., n=12). The perfusion medium was obtained as follows: A rabbit was killed by an intravenous injection of pentobarbital (200 mg), the chest cavity was opened, heart and lungs were removed and approximately 100 ml of blood was collected and heparinized (1500 I.U. per 100 ml). The blood was diluted with an equal volume of Krebs-Ringer bicarbonate buffer (pH 7.4), containing bovine serum albumin (4 mg per 100 ml) and glucose (5 mM).

For perfusion of the testis a rabbit was heparinized and anaesthetized with Hypnorm (Philips Duphar, 5-10 mg Fluanison/kg bodyweight). One testis was removed and freed from the caput epididymis and surrounding fat tissue. An incision of approximately 5 mm was made into the testis capsule, parallel with the main artery. This artery was dissected free from the tissue and was cannulated with a nr. 27 needle (Becton-Dickinson) with the aid of a binocular dissecting microscope. The testis was perfused for one minute with Krebs-Ringer bicarbonate buffer (pH 7.4). Subsequently the cauda epididymis was tied to the needle in such a way that the testis could hang unsupported on the needle. The testis was brought into the perfusion chamber and the perfusion was started (within 8-10 minutes after removal from the rabbit).

The perfusion apparatus was essentially the same as described by Ewing and Eik-Nes[55] and a modification from that of VanDemark and Ewing[56] (fig. 16). The temperature of the perfusion chamber and oxygenator was maintained at 37°C[57] by means of water circulating through the surrounding jacket. The blood was oxygenated with humidified oxygen (5% carbon dioxide) and was filtered through nylon gauze and circulated by a peristaltic pump (Buchler Instruments). Before entering the perfusion chamber, the blood



1 reservoir with magnetic stirrer
2 oxygenator
3 filter
4 peristaltic pump
5 pressure valve
6 bubble trap
7 organ chamber
8 distant thermometer
9 manometer
10 infusion pump

Fig. 16 Apparatus for perfusion of rabbit testis in vitro.

was filtered again and passed through a bubble trap. The temperature was measured by a NTC resistor probe placed near the testis. The pressure was regulated with a pressure valve in a by-pass tube. During the perfusion experiments it was necessary to increase the pressure gradually from 60 mm to 90 or 100 mm of mercury in order to obtain a constant flow rate. The testicular venous blood was collected for periods of 30 minutes in a calibrated cylinder placed in an ice-bath. The range of blood flow achieved in several experiments was 15 to 30 ml per 30 minutes. A non-pulsative blood flow was maintained because this presumably is also found in vivo. Waites and Moule reported that in the ram a pulse-pressure reduction occurs in the arterial coils in the spermatic cord so that the testis receives a relatively pulseless flow[58]. The glucose level in the blood was maintained by addition of a glucose solution into the reservoir every hour at a rate of 1 µmol per ml of blood per hour[56]. HCG, which was dissolved in 0.9% NaCl solution was added in amounts varying from 150 to 500 I.U. (0.63 ml) per 30 minutes. Radioactive substrates, e.g. 16-^{3}H-pregnenolone (1.3×10^{6} dpm per 0.63 ml per 30 minutes) were dissolved in 0.9% NaCl solution, containing 4% bovine serum albumin in order to decrease absorption to the polyethylene tubing[59]. These substances were introduced into the circulation with an infusion pump. At the end of the experiment the testis was immediately frozen in liquid nitrogen.

The following radioactive substrates have been used.

1. A mixture of 16-^{3}H- and 4-^{14}C-pregnenolone in order to check the stability of the 16-^{3}H-label in the steroid molecule.
2. 7α-^{3}H-17α-Hydroxyprogesterone with and without addition of HCG in order to establish the significance of the Δ^{5},3β-hydroxysteroid pathway.
3. 16-^{3}H-Pregnenolone, and addition of HCG was started after two hours of perfusion.
4. 16-^{3}H-Pregnenolone, with continuous addition of HCG.

2.2.4 Isolation and estimation of steroids

Specific radioactivities of the steroids in plasma and tissue samples were measured after isolation and purification. For estimation of the amount of steroid, the purified compounds were converted to derivatives with a high electron capturing ability, which permitted sensitive estimation after gas-liquid chromatography. Quantitative estimations of testosterone and dehydroepiandrosterone were performed in the same samples after the addition of tracer amounts of ^{14}C-labelled steroids for recovery determinations.

The testicular venous blood samples were separated into plasma and erythrocytes by centrifugation. The erythrocytes were washed twice with 10 ml of a 0.9% NaCl solution containing 0.5% bovine serum albumin and were centrifuged. Plasma and the wash fractions were combined and 4-^{14}C-testosterone (20,000 dpm) and 4-^{14}C-dehydroepiandrosterone (10,000 dpm) were added. Extraction was carried out with ethyl acetate (4x20 ml). Alkaline-ether extraction was not applied because of the possible ^{3}H-elimination from 17-oxosteroids (see paragraph 2.3.1). The combined extracts were washed twice with 5 ml of water and evaporated to dryness under reduced pressure.

Frozen testis tissue was homogenized, extracted and defatted as described in paragraph 1.2.8, after addition of ^{14}C-steroids (see above).

Fig. 17 gives an outline of the isolation and purification procedure. Details of the methods for acetylation, enzymic reduction of androstenedione and chloroacetylation of testosterone are given in paragraphs 1.2.4 and 1.2.5. Hydrolysis of steroid acetates was performed in 1.0 ml of methanol containing 0.2 ml of a 0.5M NaOH-solution, for 45 minutes at 50°C. The solution was then concentrated by evaporation to a volume of approximately 0.3 ml and extracted with ethyl acetate (3x2 ml). The ethyl acetate fraction was

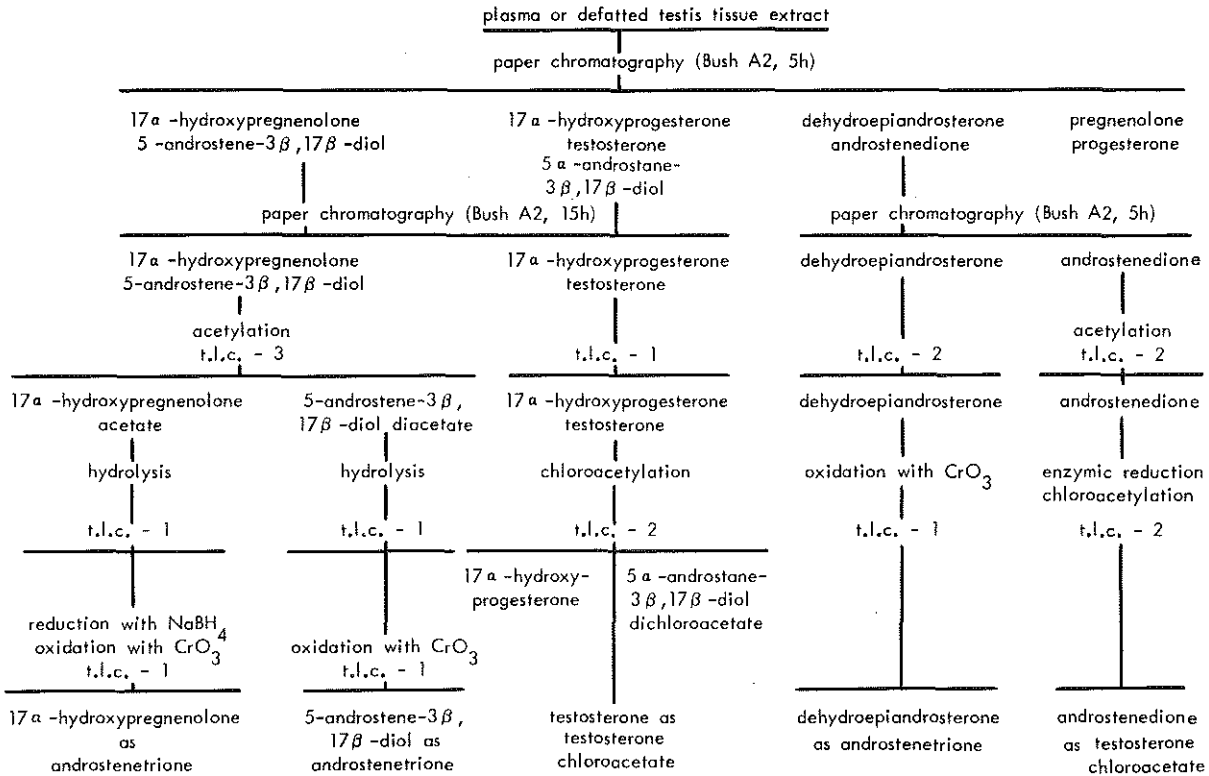


Fig. 17 Separation of steroids from extracts of spermatic vein blood and defatted testis tissue extracts.

t.l.c. 1 = t.l.c. in solvent system toluene-methanol = 9:1 (by vol.)
t.l.c. 2 = t.l.c. in solvent system toluene-ethyl acetate = 9:1 (by vol.)
t.l.c. 3 = t.l.c. in solvent system toluene-ethyl acetate = 8:2 (by vol.)

washed with water and evaporated to dryness. The 17α-hydroxypregnenolone fraction was reduced for 90 minutes by 5 mg of sodium borohydride dissolved in 0.5 ml of methanol[31]. The reaction was stopped with the addition of a drop of acetic acid and 2 ml of water. This mixture was extracted by 4x2 ml of ethyl acetate. Oxidation of steroids to androstenetrione was carried out for 10 minutes with 0.5 mg of chromiumtrioxide, dissolved in 0.1 ml of 90% acetic acid[35]. Estimation of known amounts of 17α-hydroxypregnenolone showed that the recovery of steroid with this procedure was in the range of 10 to 15%. With this procedure in the order of 10 to 30 pmoles of 17α-hydroxypregnenolone could still be detected in spermatic venous blood samples (fig. 18). The recovery of the androstenediol through the purification steps was in the order of 20%.

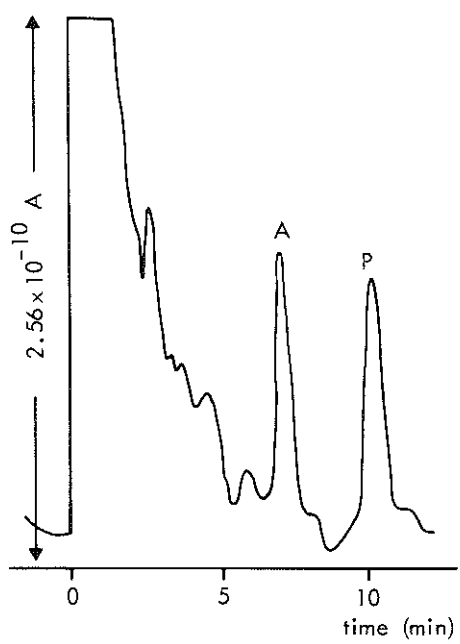


Fig. 18 Tracings obtained after gas liquid chromatography of androstenetrione (A), obtained from 17α-hydroxypregnenolone, which was isolated from a 30 minutes sample of testicular venous blood of HCG stimulated rabbit testis. Pregnenetrione (P, 33 pmoles) was added as internal standard and two-thirds of the sample was analysed.

Specific activities of the purified steroids were measured as follows. After addition of an appropriate amount of internal standard (20α-dihydroprogesterone chloroacetate or pregnenetrione[35]), a sample of the mixture was taken for measurement of ^{3}H- and ^{14}C-radioactivity and the mass of steroid was estimated in a second sample by gas chromatography with electron capture detection (paragraph 1.2.2 and reference 35). The specific activity was calculated as the ratio of the radioactivity (^{3}H dpm) and the mass of the steroid (pmoles). The amounts of testosterone and dehydroepiandrosterone in testicular venous blood and in testis tissue were calculated from the mass, estimated by gas chromatography, and the recovery of the added ^{14}C-labelled internal standard. The radiochemical purity of the dehydroepiandrosterone and 17α-hydroxypregnenolone fraction was checked by taking a sample of the corresponding androstenetrione fractions after gas chromatography.

Such dehydroepiandrosterone-androstenetrione fractions were then purified by chromatography in a Bush AII system together with 50 µg of carrier androstenetrione. Androstenetrione has a remarkable low R_f-value in this system when compared to steroids with approximately the same polarity, possibly due to enolisation of the Δ^4,3,6-di-oxogroup in the stationary methanol-water phase. This property facilitated the purification procedure. After elution from the paper, a sample of the androstenetrione was counted for ^{3}H- and ^{14}C-radioactivity. Specific activities were corrected with a factor calculated either from the shift in $^{3}H/^{14}C$ ratios or from the absolute ^{3}H-radioactivity, corrected for losses after estimation of the recovery of the carrier androstenetrione by gas chromatography. The mean drop in specific activity of androstenetrione after purification was 12% (25 samples).

The radiochemical purity of the 17α-hydroxypregnenolone fraction was evaluated by recrystallization of pooled ^{3}H-androstenetrione samples with carrier steroid (see paragraph 2.3.4). When the androstenediol fraction was purified in the

same way as described for the dehydroepiandrosterone, no decrease in specific activity was observed and therefore this fraction was regarded as radiochemically pure. The testosterone fractions were known to be pure from preliminary experiments in which an extra purification step via testosterone acetate was employed (as in paragraphs 1.2.4 and 1.2.6). The $^{3}H/^{14}C$ ratio of the testosterone acetate and of the final product testosterone chloroacetate did not significantly differ from each other:

$$\frac{^{3}H/^{14}C \text{ in testosterone chloroacetate}}{^{3}H/^{14}C \text{ in testosterone acetate}} = 0.96 \pm 0.04 \text{ (S.D., 13 samples)}$$

An assessment of reproducibility of the method for estimation of specific activities was made by dividing a purified androstenetrione sample (obtained from plasma dehydroepiandrosterone) into ten fractions, each containing approximately 60 pmoles of the steroid. The mean specific activity was found to be 98.4 ± 2.1 dpm ^{3}H per pmol (S.D., 10 estimations).

The accuracy and precision of the steroid estimations were evaluated by determination of known amount of steroids simultaneously with biological samples (table 15).

Table 15 ESTIMATION OF KNOWN AMOUNTS OF STEROIDS

Steroid	added (ng)	found (ng ± S.D.)	number of estimations
testosterone	1000 ng (3.4 nmoles)	1050 ± 80 ng	6
	100 ng	107 ± 14 ng	18
dehydroepiandrosterone	100 ng (0.34 nmoles)	103 ± 13 ng	24

2.2.5 Estimation of steroid sulfatase activity and steroid sulfates

Rat testes (2.5 g) and rabbit testis (2.9 g) were homogenized in 15 ml of 0.05M TRIS buffer (pH 7.4) using a Potter Elvejhem homogenizer. The microsomal fraction was isolated by differential centrifugation (10,000-105,000xg). Steroid sulfatase activity was determined according to Payne[20] using dehydroepiandrosterone sulfate as a substrate. Time-product relationships are given in fig. 19. Enzyme activity in rabbit testis was calculated on the basis of the conversion during 15 minutes of incubation.

Testes of rats and rabbits were washed with 0.9% NaCl solution via injection into the spermatic artery in order to remove plasma steroid sulfates (if present) from the tissue. Steroid sulfates were isolated from testis tissue and plasma by the method of Ruokonen[17], using 4-^{14}C-dehydroepiandrosterone sulfate (6,000 dpm) as internal standard. Steroid sulfates were separated from free steroids

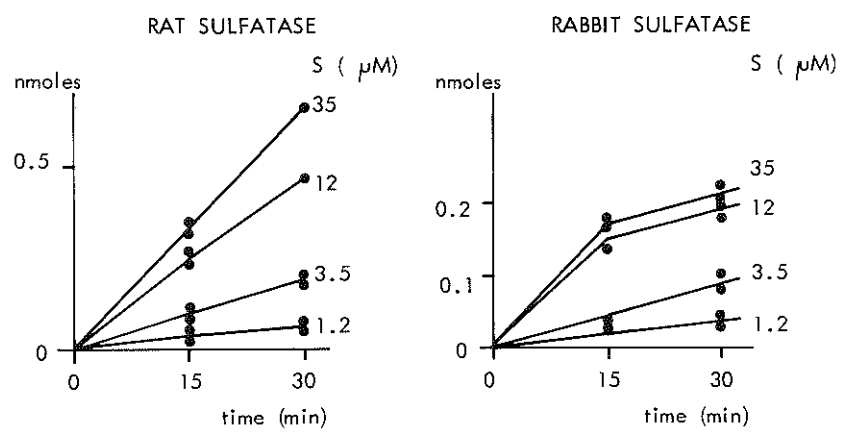


Fig. 19 Relationship between amount of product (pmoles) and incubation time (min) for the hydrolysis of ^{14}C-dehydroepiandrosterone sulfate during incubation with the 10,000 - 105,000 x g fraction of rat and rabbit testes (0.49 and 0.55 mg of protein respectively per incubation). The substrate concentrations (S) are given in the figures.

on Sephadex LH-20 columns[17]. Solvolysis was performed according to Burstein and Lieberman[60]. The free dehydroepiandrosterone and androstenediol were separated by t.l.c., oxidized by chromiumtrioxide and estimated as androstenetrione, according to the method of De Jong and Van der Molen[35]. The androstenediol concentrations in the original samples were calculated with aid of the mean recovery for ^{14}C-dehydroepiandrosterone sulfate. The sensitivity of the method was in the order of 20 pmoles of dehydroepiandrosterone sulfate. After analysis of 230 pmoles of dehydroepiandrosterone sulfate, simultaneously with the biological samples, 182 and 185 pmoles were found. After estimation of the sulfates of dehydroepiandrosterone and androstenediol in peripheral male plasma, the concentrations found were in the same order as reported previously[35,61].

The statistical significance of apparent differences between results were calculated using Student t-test.

2.3 Results

2.3.1 Elimination of tritium from metabolites of 16-^{3}H-pregnenolone*

Radioactive substrates with high specific activities were required for the perfusion experiments since these cause minimal disturbance of the endogenous steroid pools. Pregnenolone, labelled with tritium in the 7α-position could not be used, because the 7α-^{3}H-label is lost when Δ^{5},3β-hydroxysteroids are oxidized to the corresponding Δ^{4},3,6-dioxosteroids before g.l.c. estimation[35,62]. 16-^{3}H-Pregnenolone had the required high specific activity, but during the first series of experiments it appeared that the tritium label was eliminated from some of the steroid products. This loss of ^{3}H-label must have occurred either during metabolism or during the isolation procedures. Because it was important to know whether the loss of 16-^{3}H occurred during a metabolic conversion or was due to an artefact during the isolation, a mixture of 16-^{3}H- and 4-^{14}C-pregnenolone was infused into the testicular arterial blood. Elimination of tritium would then result in a decrease of the ^{3}H/^{14}C ratio in the secreted steroids, as compared to the ^{3}H/^{14}C ratio in the original pregnenolone.

The ^{3}H/^{14}C ratios of the isolated steroids, as given in table 16, are similar in the precursor pregnenolone and its metabolites. This proves that tritium was not eliminated during the metabolism of pregnenolone and that 16-^{3}H- and 4-^{14}C-pregnenolone were metabolized in the same way.

*de Bruijn, H.W.A. and van der Molen, H.J. (1973) J.Steroid Biochem. 4,85-87

Table 16 $^{3}H/^{14}C$ RATIOS IN STEROIDS isolated from the testicular venous blood after simultaneous infusion of 16-^{3}H- and 4-^{14}C-pregnenolone (21.5×10^{6} dpm ^{3}H and 0.67×10^{6} dpm ^{14}C, corresponding with 5.6 nmoles of pregnenolone per 30 minutes). Mean values and S.D. of six estimations are given.

Steroid isolated	estimated as	$^{3}H/^{14}C$
pregnenolone	pregnenolone acetate	31.9 ± 0.9
testosterone	testosterone chloro-acetate	31.7 ± 0.1
androstenedione	testosterone chloro-acetate	30.4 ± 0.6
5α-androstane-3β, 17β-diol	5α-androstane-3β,17β-diol di-chloroacetate	33.9 ± 1.0
androstenediol	4-androstene-3,6,17-trione	33.9 ± 2.9
17α-hydroxypregnenolone	4-androstene-3,6,17-trione	34.5 ± 3.3

However, after alkaline hydrolysis of isolated dehydroepiandrosterone acetate the $^{3}H/^{14}C$ ratio was 2.2 ± 0.1, thus 93% of the 16-^{3}H-label was eliminated. When in a separate experiment dehydroepiandrosterone was isolated and purified without acetylation and alkaline hydrolysis, no elimination of tritium was found ($^{3}H/^{14}C$ in dehydroepiandrosterone was 29.2 ± 0.2, in pregnenolone 27.5 ± 2.5, 4 estimations). When the androstenetrione obtained from androstenediol was further purified by ether-alkali partition[63], the ratio dropped from 33.9 to 3.3, indicating a 90% loss of tritium. More than 80% of this tritium was present in the acidified water phase. In a control experiment

the isolated and purified androstenedione was exposed to the methanolic alkali solution as used for hydrolysis. This resulted in a tritium loss of 90.4 ± 4.1% (7 estimations). Acetates of 17β-hydroxysteroids could be subjected to alkaline hydrolysis without loss of the 16-^{3}H-label. The acetic acid, used in the oxidations, did not promote the loss of the tritium at carbon atom 16.

Base-catalysed enolisation of the 17-oxogroup offers a suitable explanation for these observations. These results are in agreement with those of Fishman[64], who demonstrated that both the 16α-^{3}H- and 16β-^{3}H-label are removed from estrone benzoate by enolisation of the 17-oxogroup.

From these results it can be concluded that 16-^{3}H-pregnenolone can successfully be used in investigating steroid metabolism only if alkaline treatment of the metabolites with a 17-oxogroup is avoided.

2.3.2 Secretion of steroids by the perfused rabbit testis

Besides testosterone, the following steroids have been detected in the testicular venous blood of the perfused rabbit testis: 17α-hydroxypregnenolone, dehydroepiandrosterone, androstenediol and androstenedione. The secretion of these steroids is the result of de novo synthesis and not of a passive leakage of stored steroid from the testis (table 17). Secretion of dehydroepiandrosterone, androstene-

Table 17 LEVELS OF STEROIDS IN RABBIT TESTIS (nmoles/testis) AFTER CONTINUOUS PERFUSION WITH HCG (150-500 I.U./min) FOR 2 HOURS AND RATES OF SECRETION (nmoles/30 min, ranges are given).

Exp.	testis weight	testosterone		dehydroepiandrosterone		androstenediol*	
	g	level	secretion	level	secretion	level	secretion
1	3.9	10.0	16 - 30	0.86	1.2 - 2.5	0.30	0.49 - 0.78
2	3.5	3.8	6.4 - 7.9	0.38	1.1 - 1.7	0.13	0.30 - 0.50
3	3.7	6.1	12 - 16	1.06	1.5 - 2.3	0.19	0.41 - 0.54
4	3.1	4.9	8.4 - 16	0.61	1.1 - 1.8	0.05	0.05 - 0.12
5	3.5	7.9	9.8 - 17	0.42	0.6 - 1.7	0.18	0.13 - 0.43

*Androstenediol values were not corrected for losses, caused by the isolation procedure. The recovery of known amounts of androstenediol was in the order of 20%.

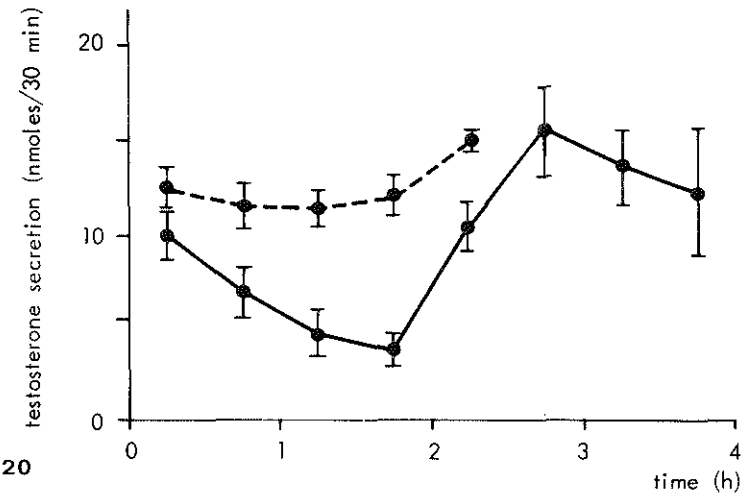


Fig. 20

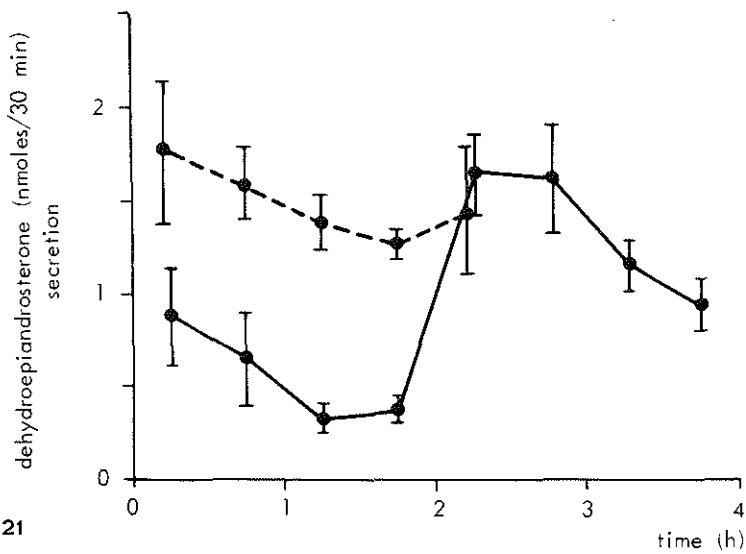


Fig. 21

Fig. **20** and **21** Time course relationship of testosterone (fig. **20**) and dehydroepiandrosterone secretion rates (fig. **21**) by the perfused rabbit testis. Mean values and S.E.M. are given (6 to 8 observations).
---------- continuous HCG infusion (150-500 IU/30 min)
——————— HCG infusion started after 2 h of perfusion

diol and other intermediates also occurs in the human testis[19,65] and dog testis[66,31] *in vivo* and is therefore not regarded as an artefact of *in vitro* perfusion experiments. The steroid secretion rate and the ability of HCG to promote steroid secretion have been used as parameters for the functioning of testes during the experiments. The secretion of testosterone and dehydroepiandrosterone with and without addition of HCG are represented in fig. 20 and 21. If addition of HCG was omitted, the secretion of these steroids decreased during the experiment, as has been observed earlier for rabbit[67] and dog testis perfusion[68]. The testosterone secretion (without administration of HCG) by rabbit testis *in vivo* was estimated as 4.3 ± 1.1 nmoles per 30 min (S.E.M., 4 estimations) and this is significantly lower ($P < 0.01$) than the secretion during the first 30 minutes of perfusion, but comparable to the secretion during the second hour. HCG infusion, starting after two hours of perfusion, increased the steroid secretion to the same values as obtained for continuously stimulated testes. Secretion of 17α-hydroxypregnenolone and androstenedione was also increased by HCG. The administered doses of HCG caused a maximally stimulated testis since increasing the infusion rate from 150 to 500 I.U. per 30 min did not result in higher testosterone secretion. In this series of experiments no correlation could be found between testis weight and steroid secretion.

From the results in fig. 20 it can be seen that testosterone secretion was constant during continuous HCG infusion and it can be expected that under these condtions a steady state incorporation of infused radioactive steroid precursor may be obtained.

2.3.3 The importance of the Δ^5,3β-hydroxysteroid pathway

It was tried to determine the relative importance of the Δ^5,3β-hydroxysteroid pathway in the intact perfused rabbit testis by infusion of tracer amounts of 7α-^{3}H-17α-hydroxyprogesterone and subsequent estimation of the specific activities of the secreted androstenedione and testosterone (table 18). The specific activities of the secreted androstenedione and testosterone indicate that a steady state condition was only achieved in the first experiment. In the other experiments the ratio of specific activities continued to decrease with the duration of the experiment and it was concluded that only a fraction of the endogenously synthesized testosterone originated from androstenedione. It was calculated that in experiment 1 about 70% and in experiment 2 more than 90% of the testosterone was synthesized from androstenediol. During HCG stimulation more than 97% of the testosterone was derived from androstenediol.

In later experiments 16-^{3}H-pregnenolone was infused, so that both Δ^5,3β-hydroxysteroids and Δ^4,3-oxosteroids were labelled. The ratio of specific activities of androstenedione over testosterone was, as might be expected, not significantly different from unity, 0.89 $\pm$ 0.12 (S.E.M., 4 perfusions).

Although the exact contribution of the Δ^5,3β-hydroxysteroid pathway could not be calculated, it is evident from these results that this route with androstenediol is the main pathway for testosterone production in rabbit testis.

Table 18 SPECIFIC ACTIVITIES (dpm/pmol) OF TESTOSTERONE AND ANDROSTENEDIONE secreted by rabbit testis during continuous infusion with 7α-^{3}H-17α-hydroxyprogesterone (1.3×10^{6} dpm and 0.06 pmoles per 30 min). In experiments 3 and 4 HCG was infused (200 I.U. per 30 min). The ratios of the specific activities of testosterone to androstenedione have been calculated.

Exp.		period (min)	testosterone secretion (nmoles/30 min)	specific activities		ratio
				testosterone	androstenedione	
1	control	0 - 30	2.4	46.5	61.5	0.76
		30 - 60	5.3	22.1	56.9	0.39
		60 - 90	4.8	22.4	54.9	0.41
		90 - 120	3.5	26.1	101	0.26
		120 - 150	4.4	27.7	79.2	0.35
2	control	0 - 30	3.8	28.3	63	0.45
		30 - 60	6.1	20.1	143	0.14
		60 - 90	3.6	18.4	264	0.07
3	HCG	0 - 30	12.4	8.9	262	0.03
		30 - 60	11.3	10.4	337	0.03
		60 - 90	6.7	12.4	753	0.02
4	HCG	0 - 30	5.5	11.5	523	0.02
		30 - 60	5.4	22.3	2290	0.01
		60 - 90	3.6	36.7	5870	0.01

2.3.4 The relationship between specific radioactivities in Δ^5,3β-hydroxysteroids and testosterone

Rabbit testes were continuously perfused with 16-^{3}H-pregnenolone and HCG. The difference in specific activities of infused pregnenolone and secreted testosterone shows that more than 99% of the mass of testosterone was derived from endogenous sources (fig. 22). The constant secretion rate of dehydroepiandrosterone and testosterone in this series of experiments was already shown in fig. 20 and fig. 21. The specific activities of these steroids in the testicular venous blood also remained constant throughout the experiments (fig. 22). Thus, a steady state situation with respect to steroid secretion was obtained. The equilibrium between endogenous steroid production and conversion of ^{3}H-pregnenolone is also demonstrated by comparison of the specific activities of steroids in the last blood samples with those of the testis tissue (table 19). The ratios of these

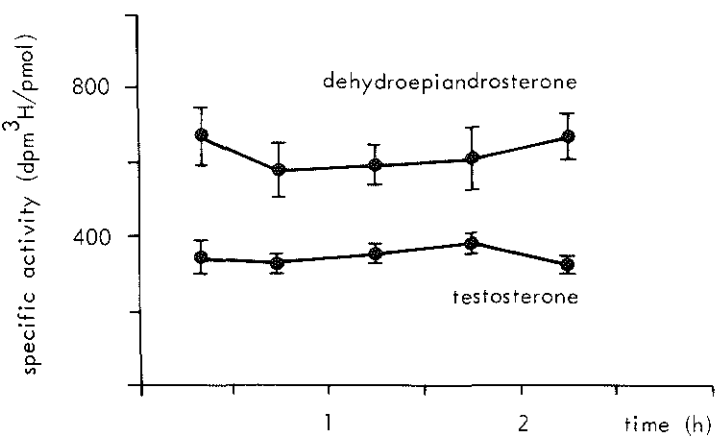


Fig. 22 Specific activities of testosterone and dehydroepiandrosterone secreted by rabbit testis in vitro, during continuous infusion of 16-^{3}H-pregnenolone (specific activity 46x10^3 dpm per pmol). Mean values and S.E.M. are given (5 or 6 observations). Continuous administration of HCG (150-500 IU per 30 min) has been applied (same series of experiments as in fig. 20 and 21).

Table 19 SPECIFIC ACTIVITIES OF STEROIDS IN SPERMATIC VEIN BLOOD AND IN TESTIS TISSUE. The mean ratios of the specific activity in the last blood sample over the specific activity in the testis tissue are given, together with S.E.M. and number of observations (n).

Steroid	ratio		S.E.M.	n
testosterone	1.16	±	0.08	6
androstenedione	0.93	±	0.15	4
androstenediol	1.54	±	0.41	6
dehydroepiandrosterone	1.55	±	0.42	5

specific activities did not significantly differ from unity. The achievement of a steady state situation in a relatively short time is apparently favoured by the short "turn-over time" (10-20 min) of steroids in the testis, as can be concluded from the results in table 17.

The ratio of specific activities in product and precursor was calculated. Specific activities of a particular steroid, isolated from different perfusions after the same time period, showed a relatively large variation and therefore this ratio was not calculated from the mean values of specific activities. Hence, the ratio was calculated from the specific activities in product and precursor in each sample. Mean ratios and their standard errors were plotted against the time (fig. 23).

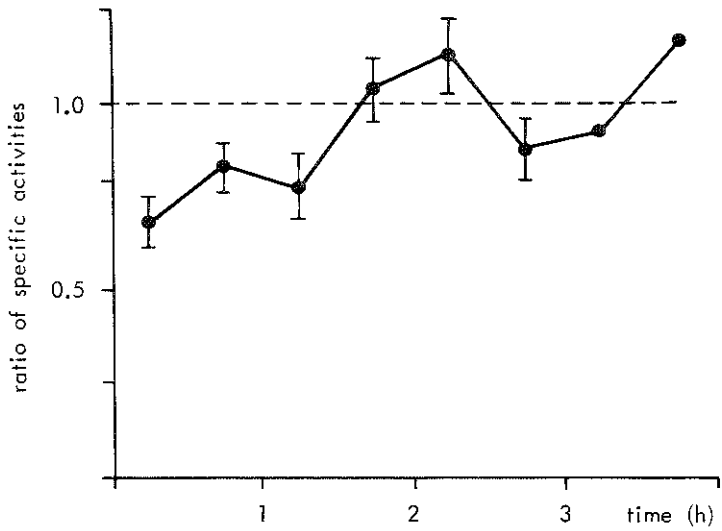


Fig. 23 Time course relationship of ratios of specific activities of steroids in testicular venous blood (same series of experiments as in fig. 22).

$$\text{ratio} = \frac{\text{specific activity of testosterone}}{\text{specific activity of androstenediol}}$$

Mean values and S.E.M. are given (4 to 6 observations, except at $3\frac{1}{2}$ and 4 h.).

During perfusion with ^{3}H-pregnenolone both androstenediol and androstenedione were labelled. These two steroids are considered as the only direct precursors of testosterone and since androstenediol is by far the most important precursor during HCG administration (see paragraph 2.3.3), the specific activities of this precursor and testosterone should equal each other. After 30 to 60 min of perfusion the ratio of these specific activities did not differ significantly from unity (fig. 23), thus the techniques used appeared to be reliable and another proof for the presence of steady state conditions was obtained.

Surprisingly after 30 minutes of perfusion the specific activity of androstenediol in the venous blood was significantly lower ($P < 0.025$) than that of dehydroepiandrosterone (fig. 24). The ratio of specific activity of product over precursor was in the order of 0.6. Because the specific activities of steroids in spermatic venous blood were

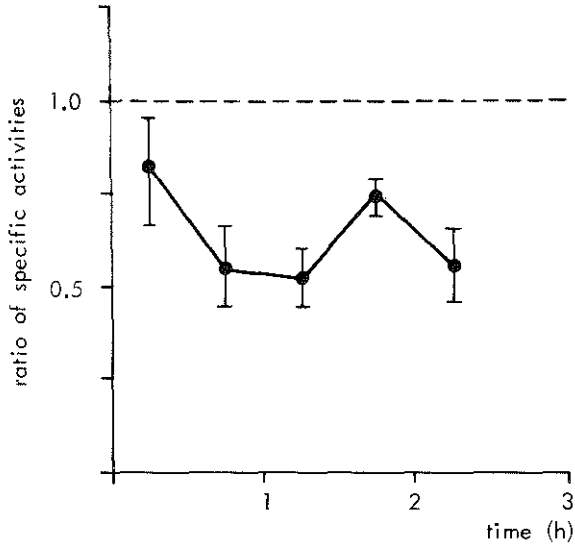


Fig. 24 Time course relationship of ratios of specific activities of steroids in testicular venous blood (same series of experiments as in fig. 22).

$$\text{ratio} = \frac{\text{specific activity of androstenediol}}{\text{specific activity of dehydroepiandrosterone}}$$

Mean values and S.E.M. are given (4 observations).

not significantly different from the specific activities of the same steroids in testis tissue (table 19) these results indicate that approximately 40% of the androstenediol might have originated from other sources than dehydroepiandrosterone. Since the testosterone secretion rate exceeded 10 nmoles/30 min (fig. 20), this unknown source of androstenediol may account for approximately 4 nmoles/30 min. It is evident that the tissue pool of unlabelled androstenediol was too small to deliver this amount (table 17) and therefore this ratio cannot be explained by the absence of steady state conditions. These data can only suggest that a second, yet unknown pathway results in de novo synthesis of androstenediol. This pathway should also by-pass 17α-hydroxypregnenolone, because the specific radioactivity of dehydroepiandrosterone nearly equals that of 17α-hydroxypregnenolone (see below).

Table 20 RECRYSTALLIZATION OF THE ^{3}H-ANDROSTENETRIONE, ORIGINATING FROM ^{3}H-17α-HYDROXYPREGNENOLONE. Fractions, isolated from the testicular venous blood of one perfusion experiment were pooled. After addition of 30 mg of androstenetrione, recrystallization was performed from the following solvent mixtures: 1. methanol-water. 2. acetone-water. 3. cyclohexane-ethyl acetate. The radioactivity in the samples was estimated with a counting error of 6% or less. The purity was calculated from the change in specific activities.

Perfusion nr.	nr. crystallization	specific activities (dpm ^{3}H/mg)		mean radiochemical purity of androstenetrione
		crystals	mother liquor	
1	0	519		
	1	438	872	
	2	375	574	
	3	327	-	≤ 63%
2	0	432		
	1	284	551	
	2	245	245	
	3	246	-	56%
3	0	281		
	1	164	594	
	2	119	259	
	3	110	131	39%
4	0	199		
	1	178	375	
	2	168	194	
	3	176	-	87%

Table 21 RELATIONSHIP BETWEEN THE SPECIFIC ACTIVITIES OF 17α-HYDROXYPREGNENOLONE AND DEHYDROEPIANDROSTERONE, secreted by the rabbit testis after infusion with ^{3}H-pregnenolone. The values for 17α-hydroxypregnenolone are corrected for the radiochemical impurities, as shown by the recrystallizations of the pooled fractions (table 20).

The ratios = $\frac{\text{specific activity of dehydroepiandrosterone}}{\text{specific activity of 17α-hydroxypregnenolone}}$ are given.

Perfusion experiment	time period min	specific activity (dpm/pmol)		ratio
		17α-hydroxypregnenolone (corrected)	dehydroepiandrosterone	
1	0 - 30	1042	928	0.89
	30 - 60	1044	819	0.78
	60 - 90	1238	779	0.63
	90 - 120	921	969	1.05
2	0 - 30	--	462	--
	30 - 60	1000	511	0.51
	60 - 90	592	355	0.59
	90 - 120	447	403	0.90
3	0 - 30	628	513	0.82
	30 - 60	317	453	1.42
	60 - 90	681	520	0.76
4	0 - 30	982	954	0.97
	30 - 60	608	437	0.72
	60 - 90	779	610	0.78
				mean ratio 0.84 ± 0.07 (S.E.M., 4 perfusions)

A preliminary experiment showed that the specific activity of 17α-hydroxypregnenolone was higher than that of dehydroepiandrosterone, however, the ^{3}H-androstenetrione, coming from 17α-hydroxypregnenolone, was radiochemically impure. Because of the small amount of radioactivity (1500 to 4000 dpm ^{3}H) in these fractions it was not possible to perform repeated recrystallizations with added androstenetrione. Therefore androstenetrione samples from one perfusion experiment were pooled after mass determination with g.l.c.. These combined samples were subsequently recrystallized with authentic androstenetrione (table 20). The found mean radiochemical purity of the combined sample was used to correct the specific activities of 17α-hydroxypregnenolone and these values were compared with the specific activities of dehydroepiandrosterone (table 21).

Although the mean value for the ratio of specific activities 0.84 $\pm$ 0.07 (S.E.M.) differs from unity ($0.025 < P < 0.05$), it is questionable if during HCG administration any dehydroepiandrosterone comes from other sources than 17α-hydroxypregnenolone. This small difference in specific activities may be caused by an error in estimating the average specific activity because complete radiochemical purity was probably not obtained in all the recrystallized fractions.

2.3.5 Steroid sulfatase and steroid sulfates

In order to explain a possible contribution to androstenediol synthesis from a steroid sulfate source, the presence of steroid sulfatase and steroid sulfates in the rabbit testis was investigated. The rabbit testis was found to have steroid sulfatase activity (fig. 25) with an apparent K_m-value in the same range as had been measured for rat testis[25,26]. The maximal conversion rate, as measured for dehydroepiandrosterone sulfate, was 1 nmol per 30 minutes per mg of microsomal protein.

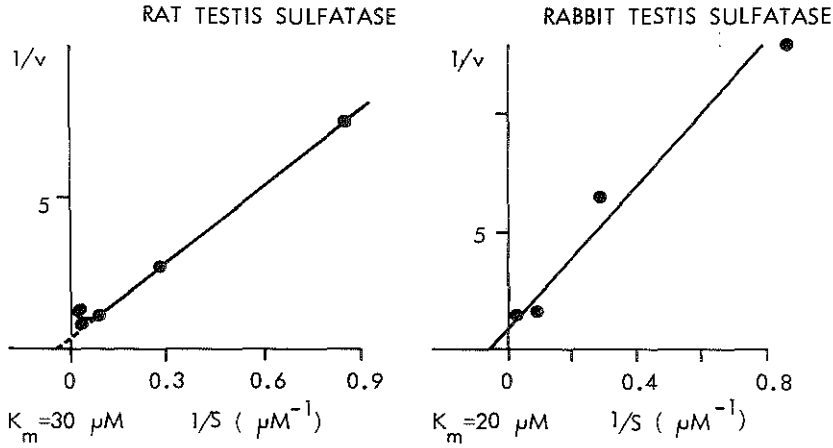


Fig.25 Steroid sulfatase activity in the 10,000-105,000 x g fraction of rat and rabbit testis, represented in a Lineweaver-Burk plot. Velocity is expressed as nmoles dehydroepiandrosterone sulfate hydrolysed during 30 minutes per mg of protein.

The testes and peripheral plasma from rabbits were analysed for the sulfates of androstenediol and dehydroepiandrosterone. The sensitivity of the method used was 20 pmoles. In the tissue both steroid sulfates were not detectable. In plasma the concentration of dehydroepiandrosterone sulfate was too low to be estimated but the level of androstenediol monosulfate was found to be 1.7 $\pm$ 0.8 nmoles/100 ml plasma (S.E.M., 4 estimations).

2.4 Discussion and conclusions

The relative importance of the Δ^5,3β-hydroxysteroid pathway and androstenediol for steroid biosynthesis when compared with the Δ^4,3-oxosteroid pathway

Rosner et al.[32] have reported that androstenediol contributes significantly to the testosterone production in rabbit testis. They came to this conclusion after incubating rabbit testis homogenates with ^{3}H-dehydroepiandrosterone and ^{14}C-17α-hydroxyprogesterone. From their data it can be derived that about one third of the labelled testosterone was synthesized via androstenediol. The results of the present perfusion experiments with tracer amounts of ^{3}H-17α-hydroxyprogesterone indicated that 70% or more of the testosterone was derived from androstenediol. These latter results may well give a better quantitative impression of the role of androstenediol in vivo because the conditions used are more physiological. It is likely that the role of androstenediol in the human testis has also been underestimated because the data were also obtained from in vitro incubation[33,40].

Possible alternative pathways in the biosynthesis of Δ^5,3β-hydroxysteroids in rabbit testis

In HCG stimulated rabbit testis dehydroepiandrosterone was almost exclusively derived from 17α-hydroxypregnenolone (see paragraph 2.3.4). This observation is consistent with the conclusion obtained by Burstein and Gut[13] for adrenal tissue. Extensive purification of the 17α-hydroxypregnenolone fraction appeared to be necessary before radiochemical

purity was obtained. The large difference in specific activities of 17α-hydroxypregnenolone and dehydroepiandrosterone found in perfusion experiments with dog testis[31] may thus be explained by impurities in the 17α-hydroxypregnenolone fractions.

The difference between the specific activities of dehydroepiandrosterone and androstenediol during HCG administration may indicate the presence of a second, yet unknown biosynthetic route for androstenediol, which by-passes 17α-hydroxypregnenolone and possibly also pregnenolone. Because of the limited number of perfusion experiments in which androstenediol has been measured, these results need verification before further conclusions are drawn. Furthermore, the method used is an indirect method for demonstrating the existence of precursors other than pregnenolone and the second direct precursor of androstenediol should be found. Nevertheless, this result gave rise to some speculation. If cholesterol hydroperoxide is involved in the side-chain cleavage reaction[69,70], unexpected rearrangements may result in a direct degration of cholesterol to androstenediol, although the formation of a C_8-fragment during the cholesterol side-chain cleavage reaction has never been shown to occur[13].

An isotope effect of the tritium on reaction rates is possible during steroid metabolism[71], but can be excluded for the metabolism of 16-^{3}H-pregnenolone, because the perfusion experiments with 16-^{3}H- and 4-^{14}C-pregnenolone demonstrated a constant $^3H/^{14}C$ ratio in the products.

Steroid sulfates as possible precursors

Precursor steroids[14,65] in the peripheral blood can, when present, be converted to testosterone in the testis. Peripheral blood plasma of rabbits was shown to contain some androstenediol monosulfate (1.6 nmoles/100 ml),

although only minute quantities of free steroids were found (see paragraph 2.3.5). As demonstrated, rabbit testes contain steroid sulfatase activity. However, steroid sulfates penetrate only slowly into intact cells[72], and the quantity of androstenediol monosulfate which passes through the testis (about 0.3 nmoles/30 min) is insufficient to serve as a quantitatively important source for the androstenediol.

Rabbit testes, at least unstimulated testes, virtually lack the presence of androstenediol sulfate and dehydroepiandrosterone sulfate and therefore steroid biosynthesis via the Δ^5,3β-hydroxysteroid sulfate pathway is insignificant. In this respect there is a marked difference with the human testis, which contains much higher levels of Δ^5,3β-hydroxysteroid sulfates than the free Δ^5,3β-hydroxysteroids[17]. More recent evidence indicates, however, that steroid sulfates are preferentially synthesized from the corresponding free steroids[73,74] and can merely be regarded as side-products of androgen biosynthesis.

It is concluded that during perfusion of rabbit testis with HCG:

- the major part of testosterone was synthesized via the Δ^5,3β-hydroxysteroid pathway and androstenediol.
- dehydroepiandrosterone was almost exclusively derived from 17α-hydroxypregnenolone.
- the ratio of specific activities of androstenediol to dehydroepiandrosterone was 0.6 and this may indicate the presence of an unknown source of androstenediol. Androstenediol sulfate did not contribute significantly to testosterone biosynthesis.

2.5 References

1 Nakano,H., Sato,H. and Tamaoki,B.(1968)Steroids 12,291-298
2 Nakano,H. and Tamaoki,B.(1969)Arch.Biochem.Biophys. 129, 771-773
3 Matsumoto,K. and Samuels,L.T.(1969)Endocrinology 85,402-409
4 Milewich,L. and Axelrod,L.R.(1972)Arch.Biochem.Biophys. 153,188-192
5 Burstein,S. and Dorfman,R.I.(1962)Acta Endocrinol. 40,188-202
6 Gual,C., Lemus,A.E., Kline,I.T., Gut,M. and Dorfman,R.I. (1962)J.Clin.Endocrinol.Metab. 22,1193-1195
7 Jungmann,R.A.(1968)Biochim.Biophys.Acta 164,110-123
8 Jungmann,R.A.(1968)Steroids 12,205-214
9 Burstein,S., Zeemoscianyk,H., Co,N., Adelson,M., Prasad,D.S.M., Greenberg,A. and Gut,M.(1971)Biochim.Biophys.Acta 231,223-232
10 Hochberg,R.B., Mickan,H. and Lieberman,S.(1971)Biochim. Biophys.Acta 231,208-222
11 Shimuzu,K.(1966)in "Dynamics of Steroid Hormones"(Pincus,G., Nakano,T. and Tait,J.F.,eds),pp.481-492,Academic Press,New York
12 Burstein,S., Kimball,H.L., Chaudhuri,N.K. and Gut,M. (1968) J.Biol.Chem. 243,4417-4425
13 Burstein,S. and Gut,M.(1971)Rec.Progr.Hormone Res. 27,303-344
14 Baird,D., Horton,R., Longcope,C. and Tait,J.F.(1968) Perspect.Biol.Med. 3,384-395
15 Wang,D.Y. and Bulbrook,R.D.(1968)Advan.Reprod.Physiol. 3, 113-146
16 Telegdy,G., Weeks,J.W., Archer,D.F., Wiqvist,N. and Diczfalusy,E.(1970)Acta Endocrinol. 63,119-133
17 Ruokonen,A., Laatikainen,T., Laitinen,E.A. and Vihko,R. (1972)Biochemistry 11,1411-1416
18 Laatikainen,T., Laitinen,E.A. and Vihko,R.(1971) J.Clin.Endocrinol.Metab. 29,219-224
19 Laatikainen,T., Laitinen,E.A. and Vihko,R.(1971) J.Clin.Endocrinol.Metab. 32,59-64

20 Payne,A.H.(1971)Biochim.Biophys.Acta 258,473-483
21 Payne,A.H., Jaffe,R.B. and Abell,M.R.(1971) J.Clin.Endocrinol.Metab. 33,582-591
22 Payne,A.H. and Mason,M.(1965)Steroids 6,323-337
23 Payne,A.H. and Jaffe,R.B.(1970)Endocrinology 87,316-322
24 Payne,A.H., Mason,M. and Jaffe,R.B.(1969)Steroids 14, 685-703
25 Notation,A.D. and Ungar,F.(1969)Steroids 14,151-159
26 Notation,A.D. and Ungar,F.(1969)Biochemistry 8,501-506
27 Notation,A.D. and Ungar,F.(1972)Endocrinology 90,1537-1542
28 Aakvaag,A., Hagen,A.A. and Eik-Nes,K.B.(1964)Biochim. Biophys.Acta 86,622-627
29 Oertel,G.W., Wendlberger,F., Menzel,P., Treiber,L. and Knapstein,P.(1967)Eur.J.Steroids 2,299-316
30 Raheja,M.C. and Lucis,O.J.(1970)J.Endocrinol. 46,21-28
31 Van der Molen,H.J. and Eik-Nes,K.B.(1971)Biochim.Biophys. Acta 248,343-362
32 Rosner,J.M., Horita,S. and Forsham,P.H.(1964)Endocrinology 75,299-303
33 Yanaihara,T. and Troen,P.(1972)J.Clin.Endocrinol. 34, 783-792
34 Rosner,J.M. and Macome,J.C.(1970)Steroids 15,181-194
35 De Jong,F.H. and Van der Molen,H.J.(1972)J.Endocrinol. 53,461-474
36 Leymarie,P. and Savard,K.(1968)J.Clin.Endocrinol.Metab. 28,1547-1554
37 Aakvaag,A.(1969)Acta Endocrinol. 60,517-526
38 MacAulay,M.A. and Weliky,I.(1968)J.Clin.Endocrinol.Metab. 28,819-830
39 Bedrak,E. and Samuels,L.T.(1969)Endocrinology 85,1186-1195
40 Rosner,J.M. and Macome,J.C.(1970)Steroids 15,181-193
41 Braunsberg,H. and James,V.H.T.(1967)J.Clin.Endocrinol. Metab. 27,1174-1188
42 Tait,S.A.S., Tait,J.F., Okamoto,M. and Flood,C.(1967) Endocrinology 81,1213-1225
43 Bell,J.B.G., Vinson,G.P., Hopkin,D.J. and Lacy,D.(1968) Biochim.Biophys.Acta 164,412-420

44 Matsumoto,K., Mondy,Y. and Samuels,L.T.(1972)in Abstracts of Fourth Int. Congress of Endocrinology, Int.Congress Series 256,p.9,Excerpta Medica Foundation,Amsterdam
45 Deshpande,N., Jensen,V., Carson,P., Bulbrook,R.D. and Douss,T.W.(1970)J.Endocrinol. 47,231-242
46 Yamaji,T., Motohashi,K., Tanioka,T. and Ibayashi,H.(1968) Endocrinology 83,992-1000
47 Vinson,G.P. and Whitehouse,B.J.(1969)Acta Endocrinol. 61, 695-708
48 Vinson,G.P. and Whitehouse,B.J.(1969)Acta Endocrinol. 61, 709-719
49 Whitehouse,B.J. and Vinson,G.P.(1971)Acta Endocrinol. 68, 467-476
50 Zilversmit,D.B., Entenman,C. and Fishler,M.C.(1943) J.Gen.Physiol. 26,325-331
51 Tait,J.F. and Burstein,S.(1965)in The Hormones(Pincus,G., Thimann,K.V. and Astwood,E.B.,eds),Vol.5,chapter 4,Academic Press,New York
52 Zilversmit,D.B. and Wentworth,R.A.(1970)J.Lipid Res. 11, 551-557
53 Bergner,P.E.(1964)J.Theor.Biol. 6,137-138
54 Gurpide,E.(1969)J.Biol.Chem. 244,5159-5169
55 Ewing,L.L. and Eik-Nes,K.B.(1966)Can.J.Biochem. 44,1327-1344
56 VanDemark,N.L. and Ewing,L.L.(1963)J.Reprod.Fert. 6,1-24
57 Hall,P.F.(1965)Endocrinology 76,396
58 Waites,G.M.M. and Moule,G.R.(1960)J.Reprod.Fert. 1,223-229
59 Fletscher,G.L., Hardy,D.C. and Idler,D.R.(1969) Endocrinology 85,552-560
60 Burstein,S. and Lieberman,S.(1958)J.Biol.Chem. 233,331-335
61 Vihko,R.(1966)Acta Endocrinol. Suppl.109,50-64
62 Pierrepoint,C.G., Galley,J.McI., Griffiths,K. and Grant,J.K., (1967)J.Endocrinol. 38,61
63 Menini,E. and Norymberski,J.K.(1962)Biochem.J. 84,195-201
64 Fishman,J.(1966)J.Org.Chem. 31,520-525
65 Lipsett,M.B.(1971)in Proc.Third Intern.Congress on Hormonal Steroids(James,V.H.T. and Martini,L.,eds),pp.8-13,Excerpta Medica,Amsterdam
66 Eik-Nes,K.B.(1967)J.Reprod.Fert. Suppl.2,125-141
67 Ewing,L.L. and Eik-Nes,K.B.(1966)Can.J.Biochem. 44,1327-1344

68 Eik-Nes,K.B.(1971)Rec.Progr.Hormone Res. 27,517-535
69 Van Lier,J.E. and Smith,L.L.(1970)Biochim.Biophys.Acta 210, 153-163
70 Lieberman,S., Bandy,L., Lippman,V. and Robert,K.D.(1969) Biochem.Biophys.Res.Comm. 34,367-371
71 Brodie,H.J. and Kim,I.(1972)in Abstracts of Fourth Intern. Congress Endocrinology,Washington,Excerpta Medica,Intern. Congress Series 256,Abstract 23
72 Mulder,E., Lamers-Stahlhofen,G.J.M. and Van der Molen,H.J. (1972)Biochim.Biophys.Acta 260,290-297
73 Yanaihara,T. and Troen,P.(1972)J.Clin.Endocrinol. 34,793-800
74 Cooke,B.A., Cowan,R.A. and Taylor,P.D.(1970)J.Endocrinol. 47,295-307
75 Cameron,E.H.D. and Griffiths,K.(1968)J.Endocrinol. 41,327-338

Summary

Testosterone formation in the testis is regulated *in vivo* by the hypophysial hormone LH and indirectly by the negative feedback effect of androgens on the hypothalamus and hypophysis. Several steroids can inhibit the *in vitro* activity of enzymes required for steroidogenesis and it is often suggested that inhibition by steroids is of importance for the control of steroidogenesis at the cellular level *in vivo*.

The first part of this thesis describes a study of the inhibition of testicular $C_{17,20}$-lyase by 17α,20α-dihydroxy-4-pregnen-3-one, a metabolite of 17α-hydroxyprogesterone. The significance of this inhibition was evaluated for the testosterone biosynthesis in rat and rabbit testes *in vivo*. The following results were obtained.

1. Radioactively labelled progesterone and 17α-hydroxyprogesterone were metabolized to 17α,20α-dihydroxy-4-pregnen-3-one during incubation and perfusion experiments.
2. 17α,20α-Dihydroxy-4-pregnen-3-one (K_i = 9 μM) and 20α-dihydroprogesterone (K_i = 2-5 μM) inhibited $C_{17,20}$-lyase activity for the conversion of 17α-hydroxyprogesterone (K_m = 2 μM) during incubation of various rat (and rabbit) testis preparations. However, infusion of these steroids into the arterial blood (10 μM) during perfusion of rabbit testes did not inhibit $C_{17,20}$-lyase.
3. With a newly developed, sensitive method for the estimation of 17α,20α-dihydroxy-4-pregnen-3-one it was not possible to conclude if any 17α,20α-dihydroxy-4-pregnen-3-one was present in rat and rabbit testis tissue and in testicular venous blood. If present, the levels of this potential inhibitor in testis tissue were at least five times lower than the levels of 17α-hydroxyprogesterone, the substrate for the $C_{17,20}$-lyase.

4. The 20α-hydroxysteroid dehydrogenase activity of rat testis was mainly localized (97%) in the seminiferous tubules and not at the site of testosterone formation in the interstitial tissue.
5. It has therefore been concluded that 17α,20α-dihydroxy-4-pregnen-3-one has no function in the regulation of $C_{17,20}$-lyase activity and testosterone production in the testis under physiological conditions.
6. The $C_{17,20}$-lyase activity from rat testis can be competitively inhibited by 17β-ureido-1,4-androstadien-3-one (K_i is approximately 0.2 μM).

It is generally accepted that steroids are derived from cholesterol and are formed along the Δ^5,3β-hydroxysteroid pathway and the Δ^4,3-oxosteroid pathway. In the rat testis the Δ^4,3-oxosteroid pathway is the quantitatively important route for testosterone biosynthesis, while both pathways are involved in the testes of man, dog and rabbit. The role of steroid sulfates in the formation of Δ^5,3β-hydroxysteroids is unclear at present.

The second part of this thesis deals with the role of Δ^5,3β-hydroxysteroids in testosterone formation. In rabbit testis the relative importance of the Δ^4,3-oxosteroid pathway and the Δ^5,3β-hydroxysteroid pathway for testosterone production was determined and the existence of precursors of dehydroepiandrosterone and androstenediol other than pregnenolone and 17α-hydroxypregnenolone was investigated. For this purpose rabbit testes were perfused in vitro with radioactively labelled substrates and the specific radioactivities of the formed steroids in testis tissue and spermatic venous blood were measured. It was observed that:

7. alkaline treatment eliminated the tritium label from metabolites of 16-^{3}H-pregnenolone, which contained a 17-oxogroup. This elimination was prevented by modification of the isolation procedure.

8. the larger part of the testosterone was synthesized via the Δ^5,3β-hydroxysteroid pathway and was derived from androstenediol.
9. during HCG administration dehydroepiandrosterone in rabbit testis was almost exclusively derived from 17α-hydroxypregnenolone.
10. after perfusion of 16-^{3}H-pregnenolone the ratio of specific radioactivities of androstenediol to dehydroepiandrosterone in testicular venous blood was 0.6, which might reflect the presence of an unknown source of androstenediol. It is unlikely that androstenediol monosulfate contributed significantly to testosterone biosynthesis.

Samenvatting

De biosynthese van het hormoon testosteron in de testis wordt in vivo gereguleerd door het hypofysaire hormoon LH, terwijl testosteron in de perifere circulatie de sekretie van LH kan beïnvloeden. Onder in vitro kondities kunnen diverse steroiden een remmende invloed uitoefenen op de aktiviteit van enzymen, betrokken bij de steroid biosynthese. Het is niet bekend of dergelijke mogelijkheden voor inhibitie van belang zijn voor de regulatie van deze steroid biosynthese onder in vivo omstandigheden. In dit verband werd de remming van het enzym $C_{17,20}$-lyase bestudeerd.

In het eerste deel van dit proefschrift wordt een onderzoek beschreven over de remming van $C_{17,20}$-lyase aktiviteit door 17α,20α-dihydroxy-4-pregneen-3-on (17,20-OH-P) in de testis. Het enzym 20α-hydroxysteroid dehydrogenase, dat nodig is voor de vorming van 17,20-OH-P uit 17α-hydroxyprogesteron, is aanwezig in testis en daarom is onderzocht of een mogelijke remming van de $C_{17,20}$-lyase aktiviteit van belang is voor de regulatie van de testosteron synthese in de testes van ratten en konijnen in vivo. De resultaten kunnen als volgt worden samengevat:

1. Radioaktief gemerkt progesteron en 17α-hydroxyprogesteron worden tijdens inkubatie- en perfusie-experimenten omgezet in ondermeer 17α,20α-dihydroxy-4-pregneen-3-on.
2. 17α,20α-Dihydroxy-4-pregneen-3-on (K_i = 9 μM) en 20α-dihydroprogesteron (K_i = 2-5 μM) remmen de $C_{17,20}$-lyase aktiviteit (K_m = 2 μM) in inkubatie-experimenten met diverse preparaten van ratte- (en konijne-)testis. Er kon geen remming van de $C_{17,20}$-lyase aktiviteit aangetoond worden, indien deze verbindingen tijdens perfusieproeven van konijnetestes werden toegevoegd aan het arteriële bloed (10 μM).

3. Er werd een methode ontwikkeld om kleine hoeveelheden 17α,20α-dihydroxy-4-pregneen-3-on te meten. Echter, de aanwezigheid van deze potentiële remmer in testis van ratten en konijnen en in testikulair veneus bloed kon niet met zekerheid aangetoond worden. Indien 17α,20α-dihydroxy-4-pregneen-3-on in testisweefsel aanwezig is, dan is de koncentratie tenminste vijfmaal lager dan die van 17α-hydroxyprogesteron.
4. De 20α-hydroxysteroid dehydrogenase aktiviteit in rattetestis is hoofdzakelijk (97% van de aktiviteit in testis) aanwezig in de seminifere tubuli en niet in het interstitiële weefsel, waar het testosteron geproduceerd wordt.
5. Uit bovenstaande resultaten werd de konklusie getrokken dat onder fysiologische omstandigheden 17α,20α-dihydroxy-4-pregneen-3-on geen funktie kan vervullen bij de regulatie van de testosteron produktie in de testis.
6. De $C_{17,20}$-lyase uit rattetestis wordt kompetitief geremd door 17β-ureido-1,4-androstadieen-3-on (K_i is ongeveer 2 μM).

Er wordt algemeen aangenomen dat testosteron gevormd kan worden uit cholesterol via de Δ^4,3-oxosteroid route en de Δ^5,3β-hydroxysteroid route. Beide route's zijn betrokken bij de testosteron synthese in de testes van mannen, honden en konijnen, terwijl in rattetestis alleen de Δ^4,3-oxosteroid van belang is. Over de rol van steroidsulfaten bij de vorming van Δ^5,3β-hydroxysteroiden bestaat geen duidelijke indruk.

In het tweede deel van dit proefschrift wordt een onderzoek beschreven naar de bijdrage van Δ^5,3β-hydroxysteroiden tot de testosteron synthese in konijnetestis. De bijdrage via de Δ^5,3β-hydroxysteroid route is kwantitatief bepaald en de mogelijke aanwezigheid van precursors voor dehydroepiandrosteron en androsteendiol naast pregnenolon en 17α-hydroxypregnenolon is onderzocht. Konijnetestes werden geperfuseerd met radioaktief gemerkte substraten en de

specifieke radioaktiviteit van de gevormde steroiden werd bepaald. De resultaten zijn als volgt samengevat:

7. Metabolieten van 16-^{3}H-pregnenolon met een 17-oxogroep verliezen de tritium in alkalisch milieu. De methoden voor de isolatie en de bepaling van steroiden werden daarom gewijzigd.
8. Het kwantitatief belangrijkste gedeelte van het testosteron werd gesynthetiseerd via de Δ^{5},3β-hydroxysteroid route en werd rechtstreeks gevormd uit androsteendiol. Onder invloed van HCG vormde deze bijdrage 97% van het totaal gevormde testosteron.
9. Tijdens HCG toediening werd praktisch alle dehydroepiandrosteron in konijnetestis gevormd uit 17α-hydroxypregnenolon.
10. Na perfusie met 16-^{3}H-pregnenolon was de verhouding van de specifieke radioaktiviteit van androsteendiol en dehydroepiandrosteron 0,6. Dit kan wijzen op een onbekende synthese route voor androsteendiol. Het is onwaarschijnlijk dat via het androsteendiol monosulfaat een kwantitatief belangrijke bijdrage tot de testosteron produktie wordt geleverd.

Curriculum vitae

H.W.A. de Bruijn werd geboren in 1944 te Alblasserdam. In 1961 behaalde hij het diploma HBS-B aan het Christelijk Lyceum te Apeldoorn en begon hij de studie Scheikunde aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen werd afgelegd in 1965, het doctoraal examen met hoofdvak Organische Chemie in december 1967. Daarna volgde een aanstelling als wetenschappelijk medewerker bij de afdeling Biochemie II van de Medische Faculteit te Rotterdam.