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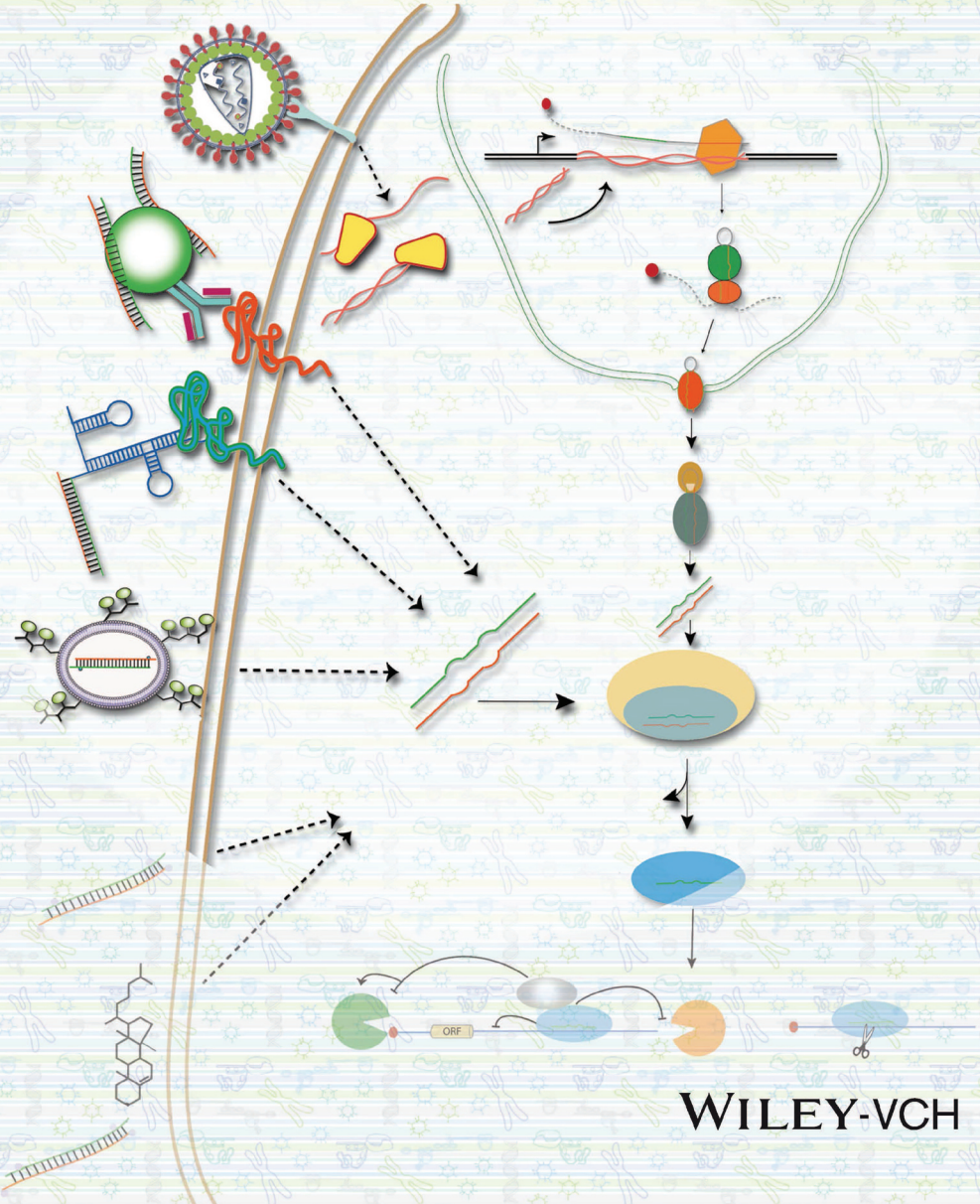
Edited by Robert A. Meyers

Translational Medicine Molecular Pharmacology and Drug Discovery



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The Editor

Dr. Robert A. Meyers
Editor-in-Chief
Ramtech Limited
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Contents

Preface

The approach we pursued in this compendium is to provide the latest insights into cutting edge methodology, approaches and results in the molecular and cellular basis of human disease as well as the discovery, evaluation, formulation and production of new drugs across the widest possible range of diseases. Two important factors are increasingly recognized in the field of translational medicine: i) despite considerable progress in the field, we still don't know enough about the mechanisms of many if not all of the critical diseases and conditions (Alzheimer's is an example), and ii) the new drug pipeline is in a shrinking mode with a concomitant decrease in R&D productivity. Our compendium aimed at capturing the *state-of-the-art* in the field and is designed to offer both answers and pathways to drug discovery and testing.

The *Biopharmaceuticals* section covers the latest developments and experimental approaches in the field from biologics extracted from living systems (*e.g.,* hormones, annexins, hemoglobin, cytokines), recombinant protein and stem cell therapies (*e.g.,* neural transplantation), as well as RNA interference in cancer therapy (nanodelivery of microRNAs), to growing organs from stem cells utilizing *in vitro* approaches to model human organogenesis producing self-organizing three-dimensional (3D) tissues so-called organoids or organ buds. This section also includes immunotherapy with autologous cells and targeted genomic approaches. Biologics approaches as applied to specific diseases such as cancer (immune checkpoint inhibitors and RNA interference), viral infections, atherosclerosis and malaria and Alzheimer's are covered in detail.

The *Drug Discovery* section provides the latest on advanced methodologies for drug discovery from cutting edge analytical techniques, *e.g.,* multidimensional HPLC and also Mass Spectrometry-Based Methods of Proteome Analysis), to drug discovery methodology, *e.g.* Hit-to-Lead and Structure-Aided Drug Design and NMR spectroscopy-based Screening as well as Neurological Biomarkers. A number of articles in this section are directed to drugs for specific diseases such as the prion family of diseases, cancer, tuberculosis, Parkinson's, Huntington's, schizophrenia, frontotemporal dementia, and Alzheimer's. *Natural Products Based Discovery* covers extraction processes from plants, animals, bacteria and associated "smart screening" methods, robotic separation with structural analysis, metabolic engineering, and synthetic biology. This section is completed with chapters on preformulation, biopharmaceutics, drug absorption, nanotechnology, pharmacokinetics and drug delivery systems design and performance including targeted drug delivery—application of physical

chemistry principles to the area of pharmacy in the design of drug molecules and drug products.

The emerging field of nanomedicine is the medical application of nanotechnology for the treatment and prevention of major ailments. The *Nanomedicine* section concerns the preparation of nanomaterials based drug delivery systems. These are nanoparticles "*which have been formulated using a variety of materials that includes lipids, polymers, inorganic nanocrystals, carbon nanotubes, proteins, and DNA origami. The ultimate goal of nanomedicine is to achieve a robust, targeted delivery of complex assemblies that contain sufficient amounts of multiple therapeutic and diagnostic agents for highly localized drug release, but with no adverse side effects and a reliable detection of any site-specific therapeutic response*"—Kim and Langer (authors of our article on Microfluidics in Nanomedicine). The Microfluidics article provides an overview of highly compatible platforms to create new nanomedicine development pipelines that include the required methodologies. Importantly, microfluidics presents a number of useful capabilities to manipulate very small quantities of samples, and to detect substances with a high resolution for a wide range of applications. Then there are articles on two exciting cutting edge nanomedicine approaches *Nanoparticle Conjugates for Small Interfering RNA Delivery* and *Quantum Dots for Biomedical Delivery Applications*, where these therapeutic approaches are discussed for cancer, hereditary disorders, heart disease, inflammatory conditions, and viral infections. In addition, quantum dots probes accumulate at tumors, due both to an enhanced permeability and retention at tumor sites, and also by the binding of antibodies to cancer-specific cell-surface biomarkers.

Larkspur, California, October 2017

Robert A. Meyers
Editor-in Chief
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Part I
Biopharmaceuticals

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1
Analogs and Antagonists of Male Sex Hormones

Robert W. Brueggemeier
The Ohio State University, Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Columbus, Ohio 43210, USA

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Keywords

Androgens
Steroid hormones responsible for the primary and secondary sex characteristics of the male, including the development of the vas deferens, prostate, seminal vesicles, and penis.

Testosterone
The C_{19} steroid hormone that is the predominant circulating androgen in the bloodstream and is produced mainly by the testis in males.

Dihydrotestosterone
The C_{19} steroid hormone that is the 5α-reduced metabolite of testosterone. It is produced in certain androgen target tissues and is the most potent endogenous androgen.

Anabolics
Compounds that demonstrate a marked retention of nitrogen through an increase of protein synthesis and a decrease in protein catabolism in the body.

Antiandrogens
Agents that compete with endogenous androgens for the hormone-binding site on the androgen receptor and thus block androgen action.

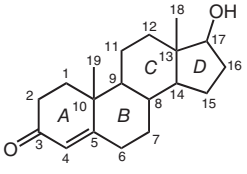
Selective androgen receptor modulators
Agents that may act as an androgen antagonist or weak agonist in one tissue, but as a strong androgen agonist in another tissue type.

5α-Reductase inhibitors
Compounds that inhibit the conversion of testosterone to its more active metabolite, dihydrotestosterone.

The steroid testosterone is the major circulating sex hormone in males and is the prototype for the androgens, the anabolic agents, and androgen antagonists. Endogenous androgens are biosynthesized from cholesterol; the majority of the circulating androgens are produced in the testes under the stimulation of luteinizing hormone (LH). The reduction of testosterone to dihydrotestosterone is necessary for androgenic actions of testosterone in many androgen target tissues such as the prostate; the oxidation of testosterone by the enzyme aromatase produces estradiol. The androgenic actions of testosterone and dihydrotestosterone are due to their binding to the androgen receptor, followed by nuclear localization, dimerization of the receptor complex, and binding to specific DNA sequences. This binding of the homodimer to the androgen response element leads to gene expression, stimulation, or repression of new mRNA synthesis, and subsequent protein biosynthesis. The synthetic androgens and anabolics were prepared to impart oral activity to the androgen molecule, to separate the androgenic effects of testosterone from its anabolic effects, and to improve on its biological activities. Novel nonsteroidal androgens, termed selective androgen receptor modulators, were developed to impart agonist activity in selective tissues. Drug preparations are used for the treatment of various androgen-deficient diseases and for the therapy of diseases characterized by muscle wasting and protein catabolism. Androgen antagonists include antiandrogens, which block interactions of androgens with the androgen receptor, and inhibitors of androgen biosynthesis and metabolism. Such compounds have therapeutic potential in the treatment of acne, virilization in women, hyperplasia and neoplasia of the prostate, and baldness.

1 Introduction

Androgens are a class of steroids responsible for the primary and secondary sex characteristics of the male. In addition, these steroids possess potent anabolic or growth-promoting properties. The general chemical structure of androgens is based on the androstane C_{19} steroid, which consists of the fused four-ring steroid nucleus (17 carbons atoms, rings A–D) and the two axial methyl groups (carbons 18 and 19) at the A/B and C/D ring junctions. The hormone testosterone (**1**) is the predominant circulating androgen and is produced mainly by the testis in males. 5α-Dihydrotestosterone (**2**) is a 5α-reduced metabolite of testosterone produced in certain target tissues and is the most potent endogenous androgen. Other endogenous androgens are produced by the adrenal gland in both males and females.



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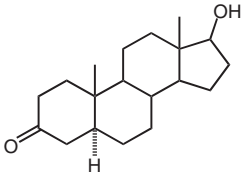


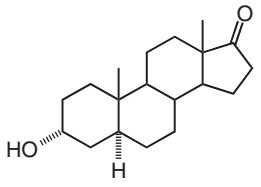
(**2**)

These two steroids and other endogenous androgens influence not only the development and maturation of the male genitalia and sex glands, but also affect other tissues such as kidney, liver, and brain. In this chapter, the endogenous androgens, synthetic analogs, various anabolic agents, and the androgen antagonists employed in clinical practice or animal husbandry in the United States and elsewhere will be discussed. Modified androgens that have found use as biochemical or pharmacological tools also are included. More extensive presentations of the topic of androgens, anabolics, and androgen antagonists have appeared in several treatises published over the past four decades [1–11].

2 Historical

The role of the testes in the development and maintenance of the male sex characteristics, and the dramatic physiological effects of male castration, have been recognized since early times. Berthold [12] was the first to publish (in 1849) a report that gonadal transplantation prevented the effects of castration in roosters, suggesting that the testis produced internal secretions exhibiting androgenic effects. However, the elucidation of the molecules of testicular origin responsible for these actions took almost another century. The first report of the isolation of a substance with androgenic activity was made by Butenandt [13, 14], in 1931. The material, isolated in very small quantities from human male urine [15], was named androsterone (**3**) [16]. A second weakly androgenic steroid hormone was isolated from male urine in 1934; this substance was named dehydroepiandrosterone (**4**) because of its ready chemical transformation and structural similarity to androsterone [17]. A year later, Laqueur [18, 19] reported the isolation of the testicular androgenic hormone, testosterone (**1**), which was 10-fold more potent than androsterone in promoting capon comb growth. Shortly after this discovery, the first chemical synthesis of testosterone was

reported by Butenandt and Hanisch [20] and confirmed by Ruzicka [21, 22].



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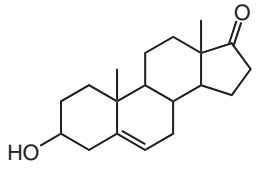
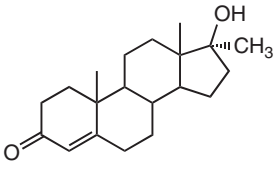


(**4**)

For many years, it was believed that testosterone was the active androgenic hormone in man. In 1968, however, research in two laboratories demonstrated that 5α-dihydrotestosterone (DHT, **2**), also referred to as stanolone, was the active androgen in certain target tissues such as the prostate and seminal vesicles, and was formed from testosterone by a reductase present in these tissues [23, 24]. Shortly thereafter a soluble receptor protein was isolated and shown to have a greater specificity for DHT and related structures [25, 26]. In general, DHT is thought to be the active androgen in tissues that express 5α-reductase (e.g., the prostate), whereas testosterone appears to directly mediate these effects in muscle and bone.

The anabolic action of the androgens was first documented by Kochakian and Murlin in 1935 [27]. In their experiments, extracts of male urine caused a marked retention of nitrogen when injected into dogs fed a constant diet. Soon afterwards, testosterone propionate was observed to produce a similar nitrogen-sparing effect in humans [28]. Subsequent clinical studies demonstrated that testosterone was capable of causing a major acceleration of skeletal growth and a marked increase in muscle mass [29–31]. This action on muscle tissue has been referred to more specifically as the myotrophic effect.

The first androgenic-like steroid used for its anabolic properties in humans was testosterone. Unfortunately, its use for this purpose was limited by the inherent androgenicity and the need for parenteral administration. 17α-Methyltestosterone (**5**) was the first androgen discovered to possess oral activity, but it too failed to show any apparent separation of androgenic and anabolic activity. The promise of finding a useful, orally effective, anabolic agent free from androgenic side effects prompted numerous clinical and biological studies.



(**5**)

3 Endogenous Male Sex Hormones

3.1 Occurrence and Physiological Roles

The hormone testosterone affects many organs in the body, its most dramatic effects being observed on the primary and secondary sex characteristics of the male. These actions are first manifested in the developing male fetus, when the embryonic testis begins to secrete testosterone. Differentiation of the Wolffian ducts into the vas deferens, seminal vesicles, and epididymis occurs under this early androgen

influence, as does the development of external genitalia and the prostate [32]. The reductive metabolism of testosterone to DHT is critical for virilization during this period of fetal development, as demonstrated dramatically in patients with a 5α-reductase deficiency [33].

At puberty, further development of the sex organs (prostate, penis, seminal vesicles, and vas deferens) is again evident and under the control of androgens. Additionally, the testes now begin to produce mature spermatozoa. Other effects of testosterone, particularly on the secondary sex characteristics, are observed; hair growth on the face, arms, legs, and chest is stimulated by this hormone during younger years. In later years, however, DHT is responsible for a thinning of the hair and recession of the hairline. At puberty, the larynx develops and a deepening of the voice occurs, the male's skin thickens, the sebaceous glands proliferate, and the fructose content in human semen increases. Testosterone influences sexual behavior, mood, and aggressiveness of the male at the time of puberty.

In addition to these androgenic properties, testosterone also exhibits anabolic (myotropic) characteristics. A general body growth is initiated, including increased muscle mass and protein synthesis, a loss of subcutaneous fat, and increased skeletal maturation and mineralization. This anabolic action is associated with a marked retention of nitrogen brought about by an increase in protein synthesis and a decrease in protein catabolism. The increase in nitrogen retention is manifested primarily by a decrease in urinary rather than fecal nitrogen excretion, and results in a more positive nitrogen balance. For example, the intramuscular administration of 25 mg testosterone propionate twice daily causes nitrogen retention to appear within 1–3 days, reaching a maximum in about 5–8 days. This reduced level of nitrogen excretion may be maintained for at least a month, and depends on the patient's nutritional status and diet [34].

Androgens influence skeletal maturation and mineralization, which is reflected in an increase in skeletal calcium and phosphorus [35]. In various forms of osteoporosis, androgens decrease urinary calcium loss and improve the calcium balance in patients; this effect is less noticeable in normal patients. Moreover, the various androgen analogs differ markedly in their effects on calcium and phosphorus balance in man [35]. Androgens and their 5β-metabolites (e.g., etiocholanolone) markedly stimulate erythropoiesis, presumably by increasing the production of erythropoietin and by enhancing the responsiveness of erythropoietic tissue to erythropoietin [36]. The effects of androgens on carbohydrate metabolism appear to be minor, and secondary to their primary protein anabolic property, but the effects on lipid metabolism seem unrelated to this anabolic property. Weakly androgenic metabolites such as androsterone have been found to lower serum cholesterol levels when administered parenterally.

3.2 Biosynthesis

The androgens are secreted not only by the testis in males, but also by the adrenal cortex in males and females, and the ovary in females. Testosterone is the principal circulating androgen and is formed by the Leydig cells of the testes. Other tissues, such as liver and human prostate, form testosterone from precursors, but this contribution to the circulating androgen pool is minimal. Since dehydroepiandrosterone and androstenedione are secreted by the

adrenal cortex and ovary, they indirectly augment the circulating testosterone pool because they can be rapidly converted to testosterone by peripheral tissues. This local production of testosterone from circulating adrenal androgens can significantly contribute to local androgen concentrations in certain tissues, such as prostate.

Plasma testosterone levels for men usually range between 6 and 11 ng ml^{-1}, and are between fivefold and 100-fold the values in females [37]. The circulating level of DHT in normal adult men is about one-tenth the testosterone level [38]. Daily testosterone production rates have been estimated at 4–12 mg for young men and 0.5–2.9 mg for young women [39]. Although attempts have been made to estimate the secretion rates for testosterone, these studies have been hampered by the number of tissues capable of secreting androgens and the considerable interconversion of the steroids concerned [40, 41].

The synthesis of androgens in the Leydig cells of the testes is regulated by the gonadotropic hormone, luteinizing hormone (LH). The other pituitary gonadotropin, follicle-stimulating hormone (FSH), acts primarily on the germinal epithelium and is important for sperm development. Both of these pituitary gonadotropins are under the regulation of a decapeptide hormone produced by the hypothalamus. This hypothalamic hormone is luteinizing hormone-releasing hormone (LHRH), also referred to as gonadotropin-releasing hormone (GnRH). In adult males, a pulsatile secretion of LHRH, and subsequently of LH and FSH, occurs at a frequency of 8–14 pulses in 24 h [42]. The secretions of these hypothalamic and pituitary hormones are, in turn, regulated by circulating testosterone and estradiol levels in a negative feedback mechanism. Testosterone will decrease the frequency and amplitude of pulsatile LH secretion [43], whereas both testosterone and a gonadal peptide, inhibin, are both involved in suppressing the release of FSH [44].

The present understanding of steroidogenesis in the endocrine organs has advanced considerably during the past four decades, based largely on initial investigations with the adrenal cortex and subsequent studies also of the testis and ovary [45]. Figure 1 outlines the following sequence of events known to be involved with steroidogenesis in the Leydig cells. LH binds to its receptor located on the surface of the Leydig cell and, via a G protein-mediated process, activates adenylyl cyclase to result in an increase in intracellular concentrations of cyclic AMP (cAMP). cAMP activates a cAMP-dependent protein kinase, which subsequently phosphorylates and activates several enzymes involved in the steroidogenic pathway, including cholesterol esterase and cholesterol side-chain cleavage [46]. Cholesterol esters (present in the cell as a storage form) are converted to free cholesterol by cholesterol esterase, and free cholesterol is translocated to the mitochondria where a cytochrome P450 mixed-function oxidase system, termed cholesterol side-chain cleavage, converts cholesterol to pregnenolone. Several non-mitochondrial enzymatic transformations then convert pregnenolone to testosterone, which is secreted.

The conversion of cholesterol (**6**) to pregnenolone (**7**) has been termed the rate-limiting step in steroid hormone biosynthesis. The reaction requires NADPH and molecular oxygen, and is catalyzed by the cholesterol side-chain cleavage complex. The latter enzyme complex is comprised of three proteins: cytochrome $P450_{SCC}$ (also called cytochrome P450 11A1); adrenodoxin; and adrenodoxin reductase. Three moles of NADPH and

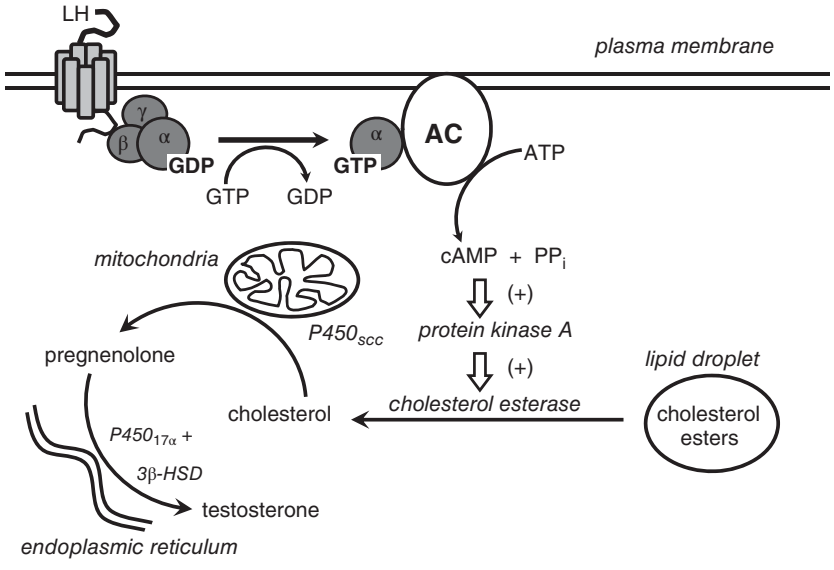


Fig. 1 Cellular events in steroidogenesis in the Leydig cell.

oxygen are required to convert one mole of cholesterol into pregnenolone (Fig. 2).

Tracer studies have shown that two major pathways known as the "4-ene" and "5-ene" pathways are involved in the conversion of pregnenolone to testosterone. Both of these pathways and their requisite enzymes are shown in Fig. 2. Earlier studies tended to favor the "4-ene" pathway, but more recent studies have disputed this view and suggest that the "5-ene" pathway is quantitatively more important in man. When Vihko and Ruokonen [47] analyzed the spermatic venous plasma for free and conjugated steroids, all intermediates of the "5-ene" pathway were identified but progesterone (**8**), an important intermediate of the "4-ene" pathway, was not found. In addition, sulfate conjugates were present in significant quantities, especially androst-5-ene-3β,17β-diol 3-monosulfate. These data strongly suggest that this intermediate and its unconjugated form constitute an important precursor of testosterone in man. This view, however, was not supported by a kinetic analysis of the metabolism of androst-5-ene-3β,17β-diol (**12**) in man [48]. Further evidence that the predominant pathway appears to be the "5-ene" pathway was provided by *in-vitro* studies in human testicular tissues [49].

Another important step is the conversion of the C-21 steroids to the C-19 androstene derivatives. Whereas, the enzymes for side-chain cleavage are localized in the mitochondria, those responsible for cleavage of the C_{17}-C_{20} bond (C_{17}-C_{20} lyase) reside in the endoplasmic reticulum of the cell. Early studies implicated 17α-hydroxypregnenolone (**9**) or 17α-hydroxyprogesterone (**10**) as obligatory intermediates in testosterone biosynthesis [50], and the C_{17}-C_{20} bond was subsequently cleaved by a second enzymatic process to produce the C-19 androstene molecule. This view of the involvement of two separate enzymes in the conversion of C-21 to C-19 steroids existed until purification of the proteins during the 1980s. The 17α-hydroxylase/17,20-lyase cytochrome

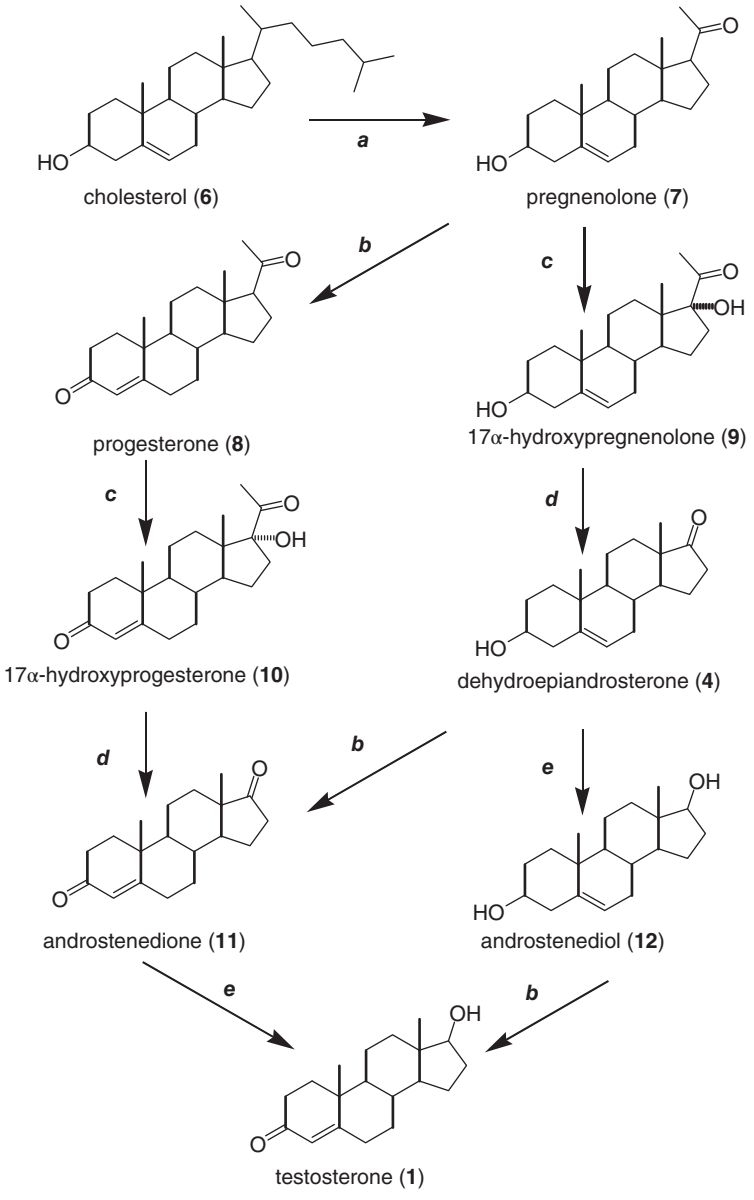


Fig. 2 Enzymatic conversion of cholesterol to testosterone. Enzymes are denoted as: (*a*) side chain cleavage; (*b*) 3β-hydroxysteroid dehydrogenase; (*c*) 17α-hydroxylase; (*d*) 17,20-lyase; (*e*) 17β-hydroxysteroid dehydrogenase.

P450 (abbreviated cytochrome P450 17 or cytochrome $P450_{17\alpha}$) was first isolated from neonatal pig testis microsomes by Nakajin and Hall [51]. Cytochrome $P450_{17\alpha}$ possessed both 17α-hydroxylase and 17,20-lyase activity when reconstituted with cytochrome P450 reductase and phospholipid. Identical full-length human cytochrome $P450_{17\alpha}$ complementary DNA (cDNA) sequences were independently isolated and reported in 1987 [52, 53]. Extensive reviews of the molecular biology, gene regulation, and enzyme deficiency syndromes have been published [46, 54].

Two additional enzymes are necessary for the formation of testosterone from dehydroepiandrosterone. The first is the 3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$-isomerase complex, which catalyzes the oxidation of the 3β-hydroxyl group to the 3-ketone and isomerization of the double bond from C_5=C_6 to C_4=C_5. Again, these processes were originally thought to involve two different enzymes, but purification of the enzymatic activity demonstrated that a single enzyme catalyzes both reactions [55]. The final enzyme in the pathway is the 17β-hydroxysteroid dehydrogenase, which catalyzes the reduction of the 17-ketone to the 17β-alcohol.

3.3 Absorption and Distribution

Although considerable research has been devoted to the biochemical mechanism of the action of natural hormones and the synthesis of modified androgens, little is known about the absorption of these substances. It is well recognized that a steroid hormone might have a high intrinsic activity but exerts little or no biological effect because its physico-chemical characteristics prevent it from reaching the site of action. This is particularly true in humans, where slow oral absorption or rapid inactivation may greatly reduce the efficacy of a drug. Even though steroids are commonly given by mouth, little is known of their intestinal absorption. One study in rats showed that androstenedione (**11**) was absorbed better than testosterone or 17α-methyltestosterone, and conversion of testosterone to its acetate enhanced absorption [56]. Results with other steroids have indicated that lipid solubility is an important factor for intestinal absorption, and this may explain the oral activity of certain ethers and esters of testosterone.

Once in the circulatory system, either by secretion from the testis or absorption of the administered drug, testosterone and other androgens will reversibly associate with certain plasma proteins, the unbound steroid being the biologically active form. The extent of this binding is dependent on the nature of the proteins and the structural features of the androgen.

The first protein to be studied was albumin, which exhibited a low association constant for testosterone and bound less-polar androgens such as androstenedione to a greater extent [57–59]. α-Acid glycoprotein (AAG) was shown to bind testosterone with a higher affinity than albumin [60, 61]. A third plasma protein to bind testosterone is corticosteroid-binding α-globulin (CBG) [62]. However, under normal physiological conditions these plasma proteins are not responsible for an extensive binding of androgens in plasma.

A specific protein termed sex hormone binding β-globulin (SHBG) or testosterone-estradiol binding globulin (TEBG) was found in plasma that bound testosterone with a very high affinity [63, 64]. The SHBG–sex hormone complex serves several functions, such as a transport or carrier system in the bloodstream, a storage site or reservoir for the hormones, and protection

of the hormone against metabolic transformations [65]. SHBG has been purified and contains high-affinity, low-capacity binding sites for the sex hormones [66]; the protein has subsequently been cloned and crystallized [66]. Dissociation constants of approximately 1×10^{-9} M have been reported for the binding of testosterone and estradiol to SHBG, and are two orders of magnitude less than values reported for the binding of the hormone to the cytosolic receptor protein [67–69]. The plasma levels of SHBG are regulated by the thyroid hormones [70] and remain fairly constant throughout adult life in both males and females [71]. SHBG is not present in the plasma of all animals [65, 72]; for example, SHBG-like activity is notably absent in the rat, and testosterone may be bound in the rat plasma to CBG.

Numerous studies have been performed on the specificity of the binding of steroids to human SHBG [65, 71–77]. The presence of a 17β-hydroxyl group is essential for binding to SHBG. In addition to testosterone, DHT, 5α-androstane-3β,17β-diol (**20**), and 5α-androstane-3α,17β-diol (**21**) bind with high affinity, and these steroids compete for a common binding site. Binding to SHBG is decreased by 17α-substituents such as 17α-methyl and 17α-ethinyl moieties and by unsaturation at C-1 or C-6. Also, 19-nortestosterone derivatives have lower affinity. The steroid-binding site and the dimerization domain of SHBG, referred to as the amino-terminal laminin G-like domain, has been crystallized and demonstrated important hydrogen bonding of the C_3 and C_{17} moieties of steroidal ligands with Ser^{42} and Asp^{65} of SHBG [78].

Another extracellular carrier protein which exhibits a high affinity for testosterone, is found in seminiferous fluid and the epididymis and originates in the testis, is called androgen binding protein (ABP) [79–81]. This protein is produced by the Sertoli cells on stimulation by FSH [82, 83], and has very similar characteristics to those of plasma SHBG produced in the liver [82].

The absorption of androgens and other steroids from the blood by target cells was usually assumed to occur by a passive diffusion of the molecule through the cell membrane. However, studies conducted during the early 1970s, using tissue cultures or tissue slices, suggested entry mechanisms for the steroids. Estrogens [84, 85], glucocorticoids [86, 87] and androgens [88–91] exhibit a temperature-dependent uptake into intact target cells, suggesting a protein-mediated process. Among the androgens, DHT exhibited a greater uptake than testosterone in human prostate tissue slices [92], and it was found that estradiol or androstenedione interfered with this uptake mechanism [93, 94]. In addition, cyproterone competitively inhibited androstenedione, testosterone and DHT entry, whereas cyproterone acetate enhanced the uptake of these androgens [91]. Little is known regarding the exit of steroids from target cells; the only reported studies have investigated the active transport of glucocorticoids out of cells [92, 93].

3.4 Metabolism

For decades, the primary function of metabolism was thought to be an inactivation of testosterone, an increase in hydrophilicity, and a mechanism to facilitate excretion of the steroid into the urine. However, the identification of metabolites of testosterone formed in peripheral tissues, as well as the potent and sometimes different biological activities of these products, has emphasized the importance of

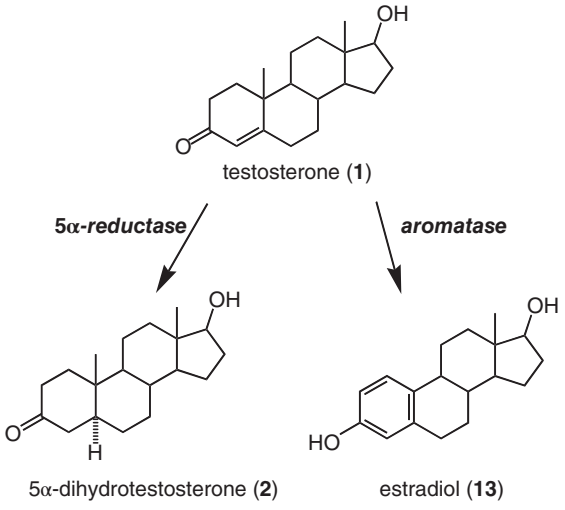


Fig. 3 Enzymatic conversion of testosterone to biologically active metabolites, 5α-dihydrotestosterone and estradiol.

metabolic transformations of androgens in endocrinology. Two active metabolites of testosterone have received considerable attention, namely the reductive metabolite 5α-dihydrotestosterone (**2**) and the oxidative metabolite estradiol (**13**) (Fig. 3).

3.4.1 Reductive Metabolism

The metabolism of testosterone in a variety of *in-vitro* and *in-vivo* systems has been reviewed [50, 94–96]. The principal pathways for the reductive metabolism of testosterone in man are shown in Fig. 4. Human liver produces a number of metabolites, including androstenedione (**11**), 3β-hydroxy-5α-androstan-17-one (**17**), 5α-androstane-3β,17β-diol (**20**), and 5α-andro-stane-3α,17β-diol (**21**) [97, 98]. In addition, cirrhotic liver was shown to produce more 17-keto-steroids than normal liver [99]. Human adrenal preparations, on the other hand, produced 11β-hydroxytestosterone as the major metabolite [100]. The intestinal metabolism of testosterone is similar to transformations in the liver [95], while the major metabolite in lung is androstenedione [101].

Studies on testosterone metabolism conducted since the late 1960s have centered on steroid transformations by prostatic tissues. Normal prostate, benign prostatic hypertrophy (BPH), and prostatic carcinoma all contain 3α-, 3β-, and 17β-hydroxysteroid dehydrogenases, and 5α- and 5β-reductases, capable of converting testosterone to various metabolites. Prostatic carcinoma metabolizes testosterone more slowly than does BPH or normal prostate [102]. On the other hand, recent studies have shown that adrenal androgens can be converted into testosterone and dihydrotestosterone in prostate cancer cells [103, 104]. K. D. Voigt *et al.* [105, 106] have performed extensive studies of *in-vivo* metabolic patterns of androgens in patients with BPH by injecting them (intravenously) with tritiated androgens 30 min before prostatectomy. Tissues from the prostate and surrounding skeletal muscle, as well as blood plasma, were then analyzed for

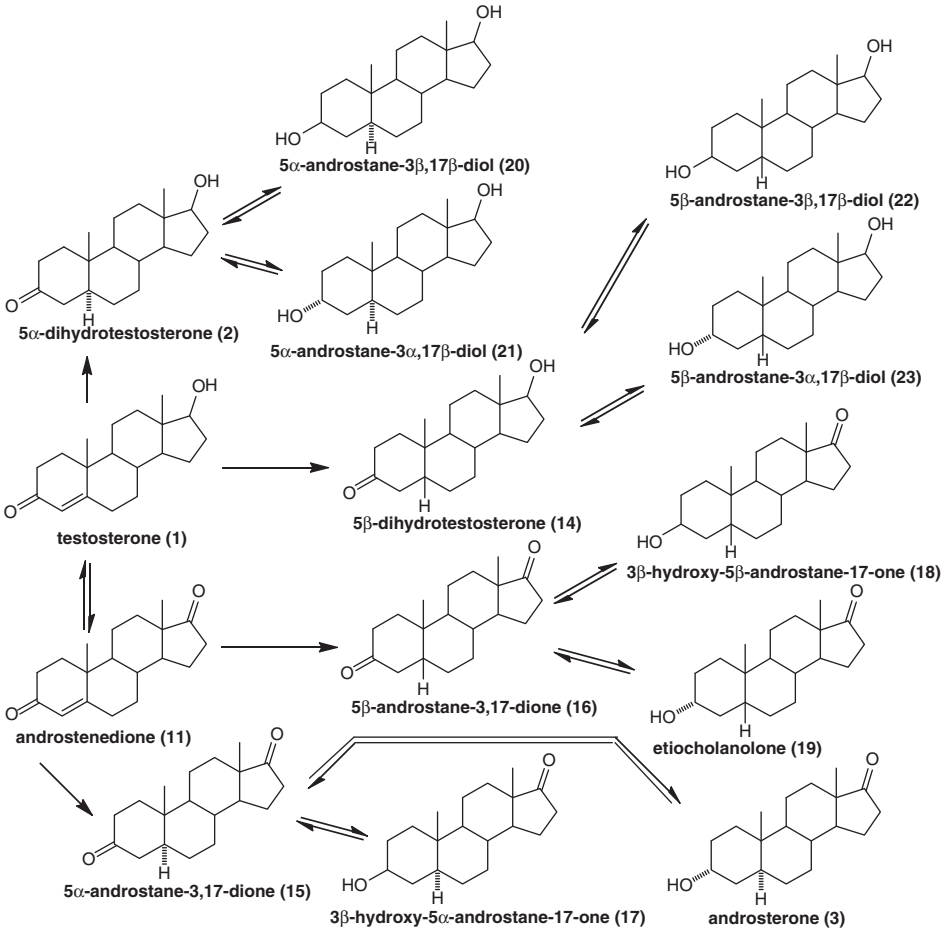


Fig. 4 Reductive metabolites of testosterone.

metabolites. The major metabolite of testosterone found in BPH tissues was DHT, with minor amounts of diols isolated. Skeletal muscle and plasma contained primarily unchanged testosterone.

Androsterone (**3**) and etiocholanolone (**19**), the major urinary metabolites, are excreted predominantly as glucuronides, and only about 10% as sulfates [37, 107]. These conjugates are capable of undergoing further metabolism. Testosterone glucuronide, for example, is metabolized differently from testosterone in man, giving rise mainly to 5β-metabolites [108]. Only a relatively small amount of the urinary 17-ketosteroids is derived from testosterone metabolism. In men, at least 67% and in women about 80% or more, of the urinary 17-ketosteroids are metabolites of adrenocortical steroids [39]. This explains why a significant increase in testosterone secretion associated with various androgenic syndromes does not usually lead to elevated levels of 17-ketosteroid excretion.

Although androsterone and etiocholanolone are the major excretory

products, the exact sequence whereby these 17-ketosteroids arise is still not clear. Studies with radiolabeled androst-4-ene-3β,17β-diol and the epimeric 3α-diol in humans showed that oxidation to testosterone was necessary before reduction of the A-ring [109]. Moreover, 5β-androstane-3α,17β-diol (**23**) was the major initial liver metabolite in rats, but this decreased with time with a simultaneous increase of etiocholanolone [110]. This formation of saturated diols agrees with studies using human liver [97] and provided evidence that the initial step in testosterone metabolism is a reduction of the α,β-unsaturated ketone to a mixture of diols, followed by oxidation to the 17-ketosteroids.

Until 1968, it was generally thought that the excretory metabolites of testosterone were physiologically inert, but subsequent studies have shown that etiocholanolone has thermogenic effects when administered to man [111]. Hypocholesterolemic effects of parenterally administered androsterone have also been described [112].

The conversion of testosterone to DHT by 5α-reductase is of major importance in the mechanism of action of the hormone, as this enzyme has been found active in the endoplasmic reticulum [113, 114] and the nuclear membrane [23, 115–120] of androgen-sensitive cells. In addition, levels of 5α-reductase are under the control of testosterone and DHT [120]; 5α-reductase activity decreases after castration and can be restored to normal levels of activity with testosterone or DHT administration [121].

Early biochemical studies of 5α-reductase were performed using a microsomal fraction from rat ventral prostate. The irreversible enzymatic reaction catalyzed by 5α-reductase requires NADPH as a cofactor, which provides the hydrogen for carbon-5 [122]. The 5α-reductase from rat ventral prostate tissues exhibited a broad range of substrate specificity for various C_{19} and C_{21} steroids [99]; this broad specificity was also observed in inhibition studies [123]. However, more detailed studies of the enzyme were limited due to the extreme hydrophobic nature of the protein, its instability upon isolation, and its low concentrations in androgen-dependent tissues [96].

Investigations of the molecular biology of 5α-reductase resulted in the demonstration of two different genes and two different isozymes of the enzyme [124–126]. The first cDNA to be isolated and cloned that encoded 5α-reductase was designated Type 1, and the second Type 2. The gene encoding Type 1 is located on chromosome 5, while the gene encoding Type 2 is located on chromosome 2. The two human 5α-reductases have approximately 60% sequence homology. The two isozymes differ in their biochemical properties, tissue location, and function [126, 127]. For example, Type 1 5α-reductase exhibits an alkaline pH optimum (6–8.5) and has micromolar affinities for steroid substrates, whereas Type 2 5α-reductase has a sharp pH optimum at 4.7–5.5, a higher affinity (lower apparent K_m) for testosterone, and is more sensitive to inhibitors than the Type 2 isozyme. The latter isozyme is expressed primarily in androgen target tissues, the liver expresses both types, and Type 1 is expressed in various peripheral tissues. Type 2 5α-reductase appears to be essential for masculine development of the fetal urogenital tract and the external male phenotype, whereas the Type 1 isozyme is primarily a catabolic enzyme. In certain cases of human male pseudohermaphroditism, mutations in the Type 2 5α-reductase gene have been observed that resulted in significant decreases in DHT levels needed for virilization [128].

3.4.2 Oxidative Metabolism

Another metabolic transformation of androgens leading to hormonally active compounds involves their conversion to estrogens. Estrogens are biosynthesized in the ovaries and placenta and, to a lesser extent, in the testes, adrenals and certain regions of the brain. The enzyme complex that catalyzes this biosynthesis is referred to as aromatase, and the enzymatic activity was first identified by Ryan [129] in the microsomal fraction from human placental tissue. The mechanism of the aromatization reaction was first elucidated during the early 1960s and continues to be the subject of extensive studies. Aromatase is a cytochrome P450 enzyme complex [130] that requires 3 mol of NADPH and 3 mol of oxygen per mole of substrate [131]. Aromatization proceeds via three successive steps, the first two of which are hydroxylations. The observation by Meyer [132] that 19-hydroxyandrostenedione (**24**) was a more active precursor of estrone (**27**) than the substrate androstenedione led to its postulated role in estrogen biosynthesis. This report, as well as numerous subsequent studies, led to the currently accepted pathway for aromatization (as shown in Fig. 5).

The first two oxidations occur at the C_{19} position, producing the 19-alcohol (**24**) and then the 19-*gem*-diol (**25**), originally isolated as the 19-aldehyde (**26**) [133, 134]. The exact mechanism of the last oxidation remains to be fully determined. The final oxidation results in a stereospecific elimination of the 1β and 2β hydrogen atoms [135–137] and a concerted elimination of the oxidized C_{19} moiety as formic acid [134]. Hydroxylation at the 2β-position was suggested as an intermediate in this final oxidation, as this substance is spontaneously aromatized to estrone [138]. However, investigations using $^{18}O_2$ and isotopically labeled steroidal intermediates failed to show any incorporation of the 2β-hydroxyl group into formic acid under enzymatic or nonenzymatic conditions [139]; neither was it demonstrated that the oxygen atoms from the first and third oxidation steps were incorporated into formic acid [140–142]. These results led to the proposal that the last oxidation step

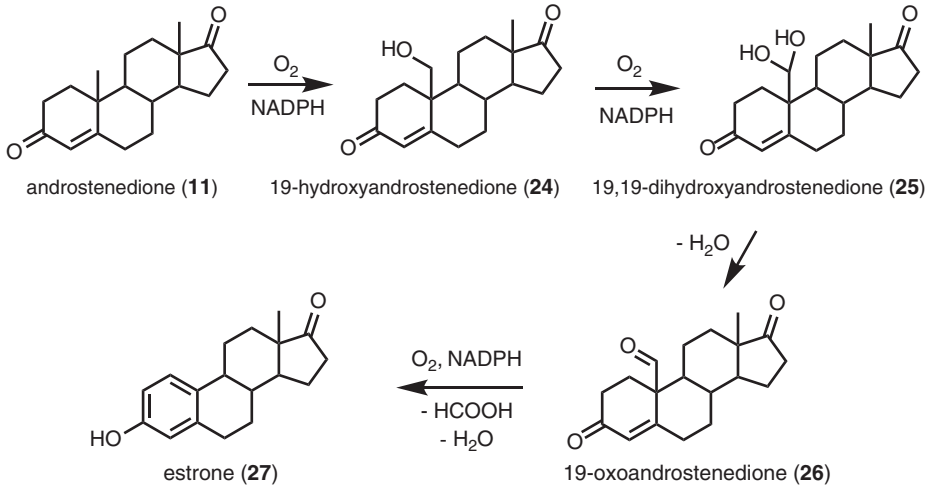


Fig. 5 Aromatization of androgens.