THE UNIVERSITY OF MANITOBA

STUDIES ON THE METABOLISM OF SOME SYNTHETIC STEROIDS IN RABBITS

by

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ABSTRACT

The *in vivo* metabolism in rabbits of some synthetic androstane derivatives was studied in order to investigate their possible transformation into active metabolites and to determine the general pattern of metabolism of monooxygenated androstane derivatives compared with mono-, bi- and tricyclic hydrocarbons.

Three synthetic steroids, 5α-androstan-3-one (I), 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (II) and 17β-hydroxy-2α-methyl-5α-androstan-3-one (III) were studied.

5α-Androstan-3-one (I), an androstane derivative with an unsubstituted D-ring, yielded the following 16- and 17-oxygenated urinary metabolites which were characterized by spectroscopic methods and comparison with authentic samples after oral administration of I; 3α- and 3β-hydroxy-5α-androstan-16-one (XXX and XXIX), 5α-androstane-3α, 16α-diol (XXVII), 5α-androstane-3β,16α-diol (XXVIII) and 5α-androstane-3β,17α-diol (XXVI). Substantially more oxidation has occurred at the 16-position compared with the more sterically hindered 17-position indicating that oxidative attack on the steroid molecule has occurred in positions furthest removed from the initial oxygen function.

The metabolism of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (II) was investigated also for the following two reasons; firstly because of the particular anabolic/androgenic properties of this substance and, secondly, as a model for the metabolism of the cyclopropane ring. The hypothesis that the activity of II may be due to its metabolic transformation into 17β-hydroxy-2α-methyl-5α-androstan-

3-one (dromostanolone, III) by initial 3β-hydroxylation to form a cyclopropanol derivative with subsequent ring opening to yield III has been tested since II and III have the same anabolic to androgenic ratios of activity. Column chromatography of the crude neutral urinary extract from the rabbit orally dosed with II yielded the following five crystalline metabolites which were identified by spectroscopic measurements. Three of these substances (2α,3α-cyclopropano-5α-androstane-4α,17α-diol, XXXVII; 2α,3α-cyclopropano-5α-androstane-4α,17β-diol, XXXVIII; 4α-hydroxy-2α,3α-cyclopropano-5α-androstan-17-one, XXXIX) were hydroxylated in the 4α-position and one in the 6α-position (2α,3α-cyclopropano-5α-androstane-6α,17β-diol, XLII). The fifth substance, 17β-hydroxy-3β-methyl-5α-androstan-2-one (XLVI) can be derived from initial hydroxylation of the cyclopropane ring at C-2 followed by ring opening and subsequent epimerization. The presence of the parent compound, II, and triol material as minor metabolites was shown by GLC and m.s. measurements.

A comparative metabolic study was carried out with dromostanolone (III) in order to determine whether any commom metabolites are formed in the rabbit. The following metabolites were isolated and identified after oral administration of III; 2α-methyl-5α-androstane-3α,17α-diol (LIV), 2α-methyl-5α-androstane-3β,17α-diol (LV), 2α-methyl-5α-androstane-3β,17β-diol (LVI) and 3α-hydroxy-2α-methyl-5α-androstan-17-one (LIII). The following triol and tetrol substances were tentatively identified as minor metabolites on the spectral evidence; 2α-methyl-5α-androstane-3α,15α,17α-triol (LVIIa), 2α-methyl-5α-androstane-3α,16α,17α-triol (LVIIIa) and 2α-hydroxymethyl-5α-androstane-3α,16α,17α-triol (LIXa). The lack of evidence for the formation of dromostanolone or its metabolites from II suggests

that this conversion is not significant in accounting for the androgenic/anabolic activity of this compound. Metabolic hydroxylation of an unactivated cyclopropane ring has not been previously reported.

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I. INTRODUCTION

Androgens constitute a class of steroids characterized by their biological effect on the primary and secondary sex characteristics of various male animals. In addition, androgens possess potent anabolic or growth promoting properties. The search for substances which possess a preponderance of one or the other of these activities has been a concern of many investigators. Theoretically, for clinical use, anabolic steroids should possess the truly anabolic activity of typical androgens, such as testosterone, but should lack all androgenic properties, such as virilizing effects. Such compounds have not been reported as yet. However, chemical modifications of testosterone have led to some synthetic compounds which show a satisfactory dissociation between anabolic and androgenic properties.

Many studies of the relationships between the androgenic and anabolic properties of synthetic 5α-androstane derivertives and their chemical structure have been carried out[1] and their in vivo and in vitro metabolism has been studied[2].

The most characteristic structural features of the compounds investigated has been the presence of an oxygen function in both the 3- and 17- positions of the steroid nucleus. Removal of the 3-oxygen function leads to increased anabolic over androgenic activity[3]. The importance of the 17-oxygen function for androgenic and anabolic activity has been generally accepted, particularly for maximum anabolic activity. However, the hydrocarbon 5α-androstane[4,5,6], and 5α-androstan-3β,16α-diol[7] both have androgenic activity, thereby indicating that a 17-oxygen function is not an essential requirement. Metabolic experiments have shown that conversion of the unsubstituted A-ring, e.g. 17β-hydroxy-17α-methyl-5α-androstane[8] and 5-androsten-17-one[9], to 3-oxygenated derivatives occurs. These results suggest that their biological activity is associated

with the more usual 3-oxygenated compounds. Therefore the relation of such metabolic alterations of androgens to their mode of action becomes significant.

The topic of this thesis is concerned with the metabolism of some synthetic androstane derivatives in order to investigate possible transformation into active metabolites and to study the general pattern of metabolism of mono-oxygenated androstane derivatives.

5α-Androstan-3-one(I) and 17β-hydroxy-2α,3α-cyclopropano-5α-androstane(II) were chosen for this investigation as representative of mono-oxygenated androstane derivatives lacking an oxygen function in the D-ring and A-ring of the steroid nucleus, respectively.

The experimental finding that conversion of the unsubstituted A-ring of the steroid nucleus to 3-oxygenated derivatives takes place and the implication of these biotransformations prompted us to investigate the metabolism of a simple androstane derivative with an unsubstituted steroidal D-ring. Therefore, the metabolism of 5α-androstan-3-one was carried out to determine whether metabolic oxidation of the D-ring occurs similarly. From the study of the metabolic pattern of simple steroid derivatives by systematic introduction of a functional group into the steroid nucleus, it may be possible to predict the general metabolic pattern, and its relationship to structure, of more complex steroids. As model substances for the study of the metabolism of alicyclic compounds the urinary excretion products by rabbits of a number of cyclohexane[10], decalin[11] and perhydroanthracene derivatives[12] has been studied. These are reviewed in detail in the following section.

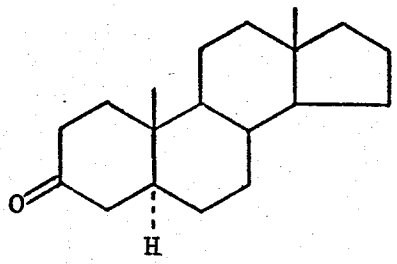
The metabolism of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane was investigated for two principal reasons. Firstly, because of the particular biological properties of this substance and, secondly, as a

model for the metabolism of the cyclopropane ring. 17β-Hydroxy-2α,3α-cyclopropano-5α-androstane has been shown to be one of the relatively few androstane derivatives without an oxygen function in the saturated A-ring which has androgenic and anabolic properties[13,14]; the anabolic effect being relatively enhanced with respect to the androgenic effect. In the examples reported in the beginning androstane derivatives having a 17 oxygen function and a saturated unsubstituted A-ring are metabolically oxidized *in vitro* and *in vivo* at the 2- and/or 3-position remote from the initial oxygen function[8,9]. Similarly, 5α-androstan-3-one is oxidized in the D-ring[15]. By analogy oxidation may be expected to occur in the A-ring of II. In particular 3β-hydroxylation may be expected to lead to the formation of a cyclopropanol derivative which could in turn undergo ring opening to yield 17β-hydroxy-2α-methyl-5α-androstan-3-one (dromostanolone, III). This compound possesses the same anabolic to androgenic ratio of activity (1.0/0.3)* as II and, therefore, may be the source of its activity. It was also of interest to study the metabolism of II since it has been stated that oxidative removal of the cyclopropane ring in II is unlikely, however, no supporting experimentation or rationale was given[13,16]. *In vivo* formation of dromostanolone may also be of significance because this substance has been used in the treatment of some breast carcinomas[17]. Therefore, a comparative metabolic study was carried out with dromostanolone, a potential metabolite of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane, in order to determine whether any common metabolites are formed in the rabbit.

In general the metabolism of the cyclopropane ring, which in recent years has become a more commonly available synthetic moiety, can be studied here in a molecule where it is potentially favourably situated for metabolic oxidation. The potential of the cyclopropane ring for hydroxylation is

significant taking into consideration that the naturally occurring triterpene, phorbol, containing a cyclopropanol function has been shown to possess cancer promoting properties[18].

The experiments were carried out by means of large scale feeding of steroids to experimental animals from which the major metabolic products can be isolated and fully characterized by the usual spectroscopic and comparison methods. Unless all possible isomers were available for comparison identification of the metabolites by chromatographic methods in conjunction with mass spectroscopy alone would not yield unequivocal results in all cases.



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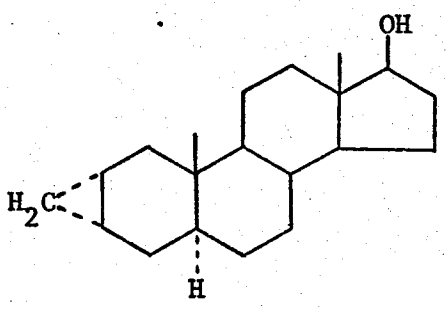
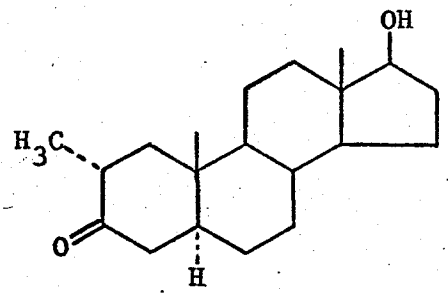


II



III

* see APPENDIX A

II. GENERAL CONSIDERATIONS

A. Drug metabolism

Drug metabolism is an important branch of science devoted to studying all aspects of the fate of foreign molecules in biological systems under *in vivo* or *in vitro* conditions. Since the investigations described as "drug metabolism" studies frequently do not include either drug substances *per se* (e.g., pesticides and the influences of adjuvants) or biotransformation of the parent compounds, a new term "xenobionics" has been introduced recently to describe the study of this field in a broader term[19]. Strictly speaking, drug metabolism refers exclusively to the chemical alterations of a drug produced by the biological environment and thus represents one aspect of the physiological disposition, or fate, of the agent, which encompasses its absorption, distribution, metabolism, and excretion.

Metabolic studies have led to the discovery of new drugs with a variety of therapeutic actions. Not only have some drugs been shown to owe their activity to metabolic products, but knowledge of drug metabolism has furnished the medicinal chemist with clues to new compounds which have more desirable absorption, excretion, metabolism and tissue distribution characteristics. Knowledge of the metabolic pathways of molecules permit one to predict what metabolites may be formed from a drug.

Most drugs or foreign compounds are metabolized in the liver, and metabolizing enzymes can occur in the soluble, mitochondrial, or microsomal fractions. The most common routes of drug metabolism involve oxidation, reduction, hydrolysis, and conjugation. Very often a drug is subjected to several competing pathways simultaneously, and the extent of formation of the various metabolites depends on the relative rates of the various interactions.

The study of metabolism in androgens is particularly important in view of the finding[20] that the truly anabolic effect of testosterone

begins only at a time when all but traces of the effective dose of the steroid have been metabolized and excreted by the body. Therefore the relation of such metabolic alterations of androgens to their mode of action and the nature of the "active forms" of testosterone and other androgens in target tissue is of significance. Some of the testosterone metabolites found in the prostate in the in vivo and in vitro experiments exhibit noticeable androgenic properties[21]. With reference to the prostate weight, it has been found that 5α-dihydrotestosterone(DHT) and 5α-androstane-3α,17β-diol are at least as active as testosterone or more so[21]. The action of testosterone could, therefore, be caused directly by the hormone, or indirectly through its transformation into metabolites. Further studies by other workers[22,23] strongly suggest that testosterone action on the ventral prostate is related to the formation of active metabolites.

The comparative relative activities of testosterone metabolites on the seminal vesicle and ventral prostate of immature male rats and capon's comb are summarized in Table 1.

Table 1. The comparative relative activities of testosterone metabolites (from Ref. 1)

Metabolites of testosterone	Testosterone=100% Relative activity (%)		
	Seminal vesicles(21)	Capon's comb(21)	Ventral prostate
Testosterone	100	100	100
Androstane-17β-ol-3-one	200	75	260
Androstane-3α,17β-diol	33	75	24
Androstane-3β,17β-diol	10	2	3
Δ^4-Androstene-3,17-dione	20	12	39
Androstane-3,17-dione	14	12	33
Δ^5-Androstene-3,17β-diol	14	3	21
Androstane-3α-ol-17-one (Androsterone)	10	10	53
Androstane-3β-ol-17-one (Epiandrosterone)	3	2	2
Δ^5-Androstene-3,17,dione	7	12	-
Δ^5-Androstene-3β-ol-17-one (Dehydroepiandrosterone)	3	16	34

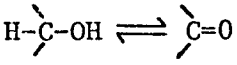
It can be seen that 17β-hydroxy-5α-androstan-3-one(DHT) is 2 to 2½ times as active as testosterone on the siminal vesicles and ventral prostate indices, but the rest of the compounds are all less active than testosterone. Thus metabolic inactivation is a very important factor to be considered in the evaluation of a drug.

B. Metabolism of Steroids: Classification

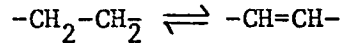
Metabolism of steroids has been the object of intensive study in biological systems of varying organizational complexity. It is believed that relatively little breakdown of the steroid ring system occurs in mammalian tissues. However, the side chain of cholesterol is selectively oxidized, probably in a stepwise fashion, leading to the formation of bile acids and various classes of steroid hormones.

The principal types of metabolic transformations of steroids are oxido-reductions, hydrolytic reactions and conjugations. The oxidations and reductions may be conveniently grouped into 5 categories according to the chemical groups involved[24].

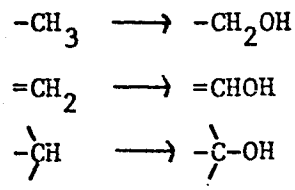
a) Interconversions of hydroxy- and keto-steroids involving both the steroid skeleton and side chain:



b) Introduction and hydrogenation of carbon to carbon double bonds:



c) Hydroxylations of 1^{o}, 2^{o} and 3^{o} carbon atoms on the steroid skeleton and side chains:



d) Epoxidations of ring unsaturated steroids:

$$-CH=CH- \longrightarrow -\overset{O}{\overset{/\backslash}{CH-CH}}-$$

e) Oxidative fission of carbon to carbon bonds. This category comprises a series of miscellaneous reactions involved in the degradation of the side chain and in the total oxidation of the steroid nucleus. The reactions result in the formation of ketones, acids and lactones. Little is known of their enzymatic mechanisms but some aspects of these reactions have been reviewed by Hayano et al.[25].

Steroids participate in two other types of reaction which are not oxidation or reductions:

f) Hydrolyses of steroid esters, glycosides and glucuronides, and

g) Conjugation reactions. These comprise conjugations of steroid alcohols to form principally sulfates and β-glucuronides.

Recently, Dorfman and Ungar[26] has attempted a classification or organization of the many diverse reactions involving more than 200 different steroids identified so far from tissue and urinary sources.

i) Hydroxylation Reaction

The number and types of steroid hydroxylations described in animal tissues and microorganisms continue to increase, and these reactions are evidently widespread in living systems. Their function in some instances is clearly related to the synthesis of key metabolic compounds, as in the case of the 11β-, 17α-, and 21-hydroxylations of the adrenal cortex which are specifically concerned with the biosysthesis of corticosteroids[25], or the hydroxylations at C-6, C-7, and C-12 which are essential to the formation of bile acids[27].

Peterson[28] has prepared the most recent compilations of steroid hydroxylations by microorganisms and has contrasted these with the analogous reactions in animal tissues. Microbial enzyme systems are capable of introducing hydroxyl groups into at least 21 and perhaps even as many as 23 sterically distinct positions on the steroid nucleus and side chains: 1α, 1β, 2β, 6β, 7α, 7β, 9α, 10β, 11α, 11β, 12α, 12β, 14α, 15α, 15β, 16α, 16β, 17α, 18, 19, 21 as well as probably 5β and 8β. Mammalian enzymes are known to hydroxylate in the following positions: 2α, 2β, 2(aromatic), 6α, 6β, 7α, 10β, 11β, 12α, 16α, 16β, 17α, 18, 19, 20, 21, 25, 26 and 27. It is by no means certain that all of these reactions are catalyzed by separate hydroxylases, and when both epimeric hydroxyl groups are formed at a single carbon atom, this may be a consequence of a single hydroxylase acting in concert with two stereospecific hydroxy steroid dehydrogenases which cause inversion of configuration via the ketonic intermediate. With the suitable deoxy steroid substrate it is likely that hydroxylation could be shown to occur at any position on the steroid molecule[26].

Steroid hydroxylations as well as the oxidative metabolism of many drugs are aerobic reactions catalyzed by enzymes "monooxygenase" which activate molecular oxygen and cause the direct incorporation of one atom of oxygen into the substrate, while the other oxygen atom is reduced to water in the presence of NADPH[29,30]. Some aspects of the internal mechanisms of the catalysis have been discussed by Talalay[24] and Tomkins[31]. Analysis of the hydroxylation reaction reveals discrete steps involving (1) oxygen activation, (2) substrate activation, if any, and oxygen transfer, and (3) regeneration of coenzymes. Except for the regeneration of NADPH, the entire sequence may occur in a single concerted process. Hayano[32] suggested that in the case of the oxygen in 11β-hydroxylation,

the substrate and NADPH are brought together to a single active site on the enzyme surface. In the proximity of a suitably oriented steroid substrate, the oxygen is activated probably by fixation to a metal followed by NADPH reduction. Momentary "stretching" of the hydrogen of the position under attack may occur, although on the basis of chemical analogy this would not be necessary, and finally the positively charged species, depicted here as OH^+, displaces the original hydrogen to complete the formation of the hydroxylated steroid. The unutilized atom of the oxygen can accept the hydrogen taken from the substrate and, together with a proton, is liberated as water. Regeneration of NADPH would occur as a final step and as a prelude to the next cycle.

The monooxygenases containing iron in the form of cytochrome P-450 as the oxygen-activating component have been the most intensively studied so far. The central problem of the activation of oxygen has not yet been solved, however, recently an "oxenoid" structure has been postulated as the active oxygen, the attacking oxygen being defined as an electrophilic particle with six valence electrons[33].

The overall reaction may be formulated as follows, (Scheme I) where A is the oxidized form and AH_2 is the reduced form of cytochrome P-450[30].

Scheme I

1. $NADPH + A + H^+ \longrightarrow AH_2 + NADP^+$
2. $AH_2 + O_2 \longrightarrow$ "active oxygen complex"
3. "active oxygen complex" + drug substrate $\longrightarrow$ oxidized drug + A + H_2O

$NADPH + O_2 + \text{drug substrate} + H^+ = NADP^+ + \text{oxidized drug} + H_2O$

Hydroxylation reactions of steroids lead to metabolites which are

usually less active than the precursors. Hydroxylation reactions may take place at a position activated in the chemical sense, such as an allylic methylene group or a position adjacent to or vinylogous to a carbonyl function. Hydroxylation of saturated carbon atoms that are inactivated in any classical sense are also important. For hydroxylation at saturated carbon, chemical analogy suggests that activation is unnecessary. Bloom[34,35] made a discovery which provided the basis for his proposed mechanism for oxidative attacks on steroidal substrates, including hydroxylation and epoxidations. An enzyme system capable of forming an axial hydroxyl function at a specific carbon of a saturated steroid could also cause the formation of an epoxide involving the same carbon atom in the corresponding unsaturated substrate. Equatorial hydroxylases did not effect a similar conversion. From this interesting correlation, it was proposed that hydroxylations proceed by electrophilic attack. Evidence compatible with the hypothesis is provided by the demonstrations of enzymatic hydroxylations at 7α[36], 11α[37], and at 11β[38] of C_{21} steroids where incoming hydroxyl groups directly replaced the hydrogens at those positions hydroxylated, with retention of configuration. In chemical systems electrophilic displacement at saturated carbon atoms has been found to occur in this way[39].

The question of substrate activation has been discussed by Ringold[40], particularly with respect to positions adjacent to or vinylogous to carbonyl functions. The suggestion has been made that in reactions at carbons 2, 6, 10, 16, 17 and 21 the substrate may undergo reaction while in an enolic state. Enolization of the substrate hydrogens on methyl or methylene groups adjacent to keto oxygens occur on the enzyme surface producing a high electron density at the positions under attack, thus aiding the incoming positively charged hydroxylating species. Maximal overlap