THE UNIVERSITY OF MANITOBA

STUDIES ON THE METABOLISM OF SOME SYNTHETIC STEROIDS IN RABBITS

by

RYUNG-SOON SONG KIM

A THESIS

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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.

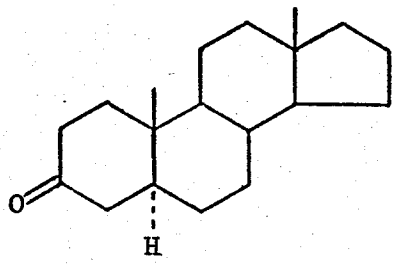
The metabolism of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane was investigated for two principal reasons. Firstly, because of the particular biological properties of this substance and, secondly, as a

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.

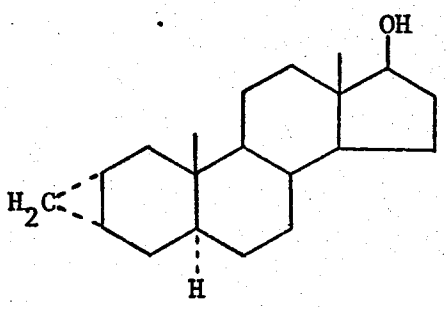
In general the metabolism of the cyclopropane ring, which in recent years has become a more commonly available synthetic moiety, can be studied here in a molecule where it is potentially favourably situated for metabolic oxidation. The potential of the cyclopropane ring for hydroxylation is

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

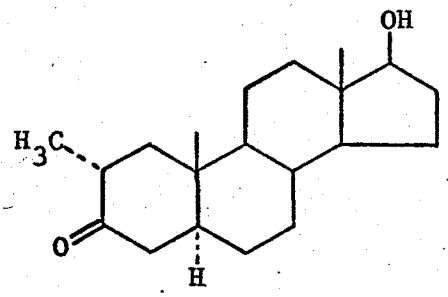
The experiments were carried out by means of large scale feeding of steroids to experimental animals from which the major metabolic products can be isolated and fully characterized by the usual spectroscopic and comparison methods. Unless all possible isomers were available for comparison identification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



I



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

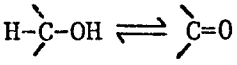
It can be seen that 17β-hydroxy-5α-androstan-3-one(DHT) is 2 to 2½ times as active as testosterone on the siminal vesicles and ventral prostate indices, but the rest of the compounds are all less active than testosterone. Thus metabolic inactivation is a very important factor to be considered in the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

B. Metabolism of Steroids: Classification

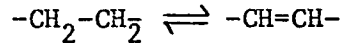
Metabolism of steroids has been the object of intensive study in biological systems of varying organizational complexity. It is believed that relatively little breakdown of the steroid ring system occurs in mammalian tissues. However, the side chain of cholesterol is selectively oxidized, probably in 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].

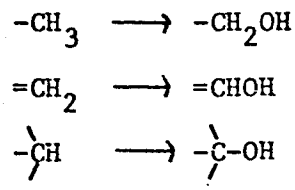
a) Interconversions of hydroxy- and keto-steroids involving both the steroid skeleton and side chain:



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

of π-electrons is expected to occur with axial attack and, thus, in the plane favorable for the 2β-, 6β-, 10β-, and 17α- configurations. In chemical systems, hydroxylations at these sites predictably predominate as compared with those at 2α, 6α, 10α, and 17β. In biological systems where enzyme specificity is the controlling factor, a valid comparison cannot be made. However, in the literature the documentation of hydroxylations effected by both microorganisms and animal tissues at these sites shows that some parallelism exists.

Recently, evidence has been presented[41a] for the involvement of enol intermediates in the cytochrome P-450 mediated hydroxylation of testosterone at the 2- and 6-positions with rat hepatic microsomes. In order to investigate further the possible role of enol intermediates in steroidal hydroxylation, the interaction and metabolism of [6β-^{2}H]testosterone with rat liver microsomes were studied by Toft[41b]. The 6β-hydroxylated product obtained did not contain any deuterium. This result is consistent with two possible reaction pathways;

a) either direct replacement of the axial 6β-deuterium with -OH, or

b) involvement of the Δ^{4}-3-keto group via an enzymically specific loss of the 6β-deuterium to form the enol V(Fig. 1) followed by hydroxylation of the enzyme-enol complex.

If the role of the enzyme is merely to induce enolization by electron withdrawal from the 3-position, it would cause only a preferential loss of the proton from the 6β-position than from the 6α-position, and not a completely specific loss. The resulting 6β-hydroxy product (VII) would then contain some deuterium. Furthermore, Gustafsson et al.[43] have reported that incubation of 5α-dihydrotestosterone with rat liver microsomes leads to products hydroxylated at positions 2β, 7α, and 16α but

not 6β. The fact that testosterone is metabolized by this system to 6β-hydroxylated products also indicates that the Δ^4-3-keto moiety plays a role in 6β-hydroxylation. The author concluded that it is also conceivable that an enzyme-enol complex could serve as an intermediate for other metabolic reactions, such as the hydroxylation of the 4,5-double bond. The mechanism of enzymic hydroxylation by microorganisms at an enolizable position has also been studied by Baba *et al*[42].

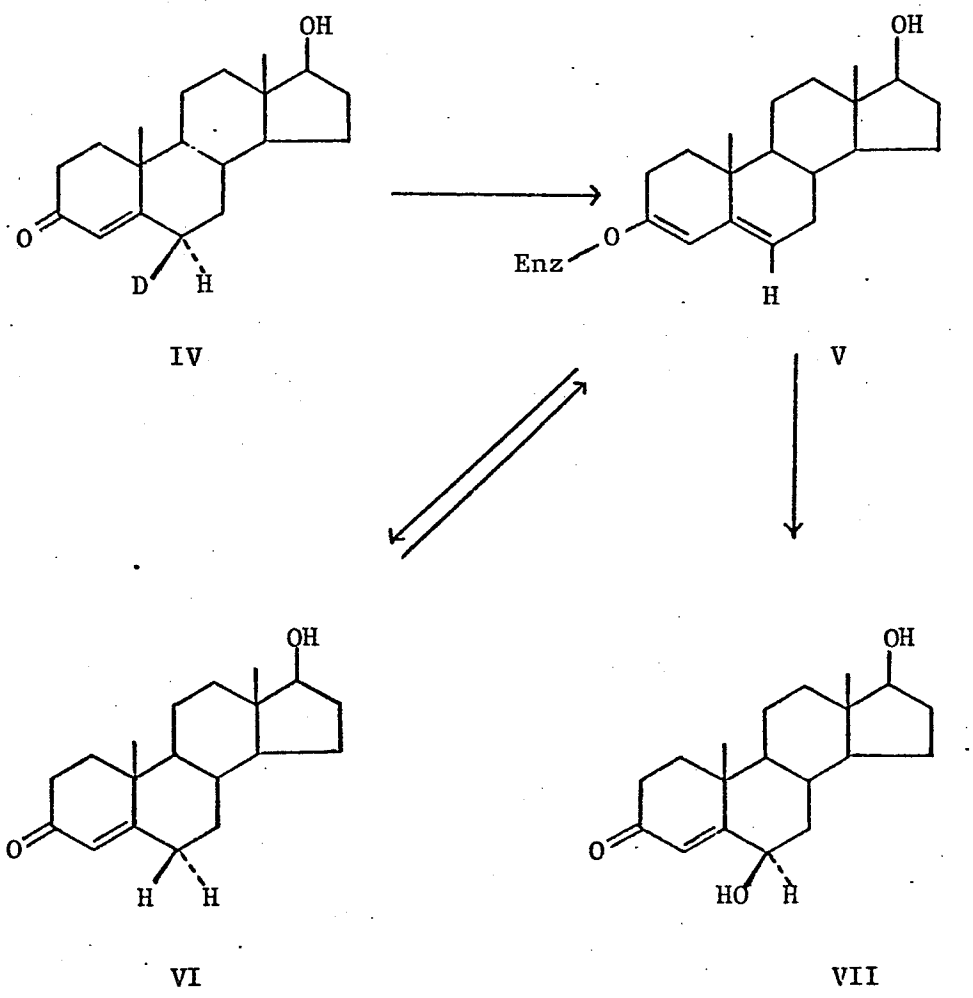
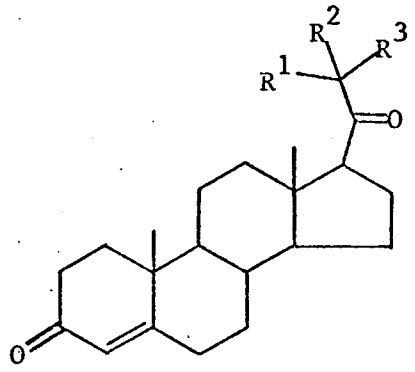


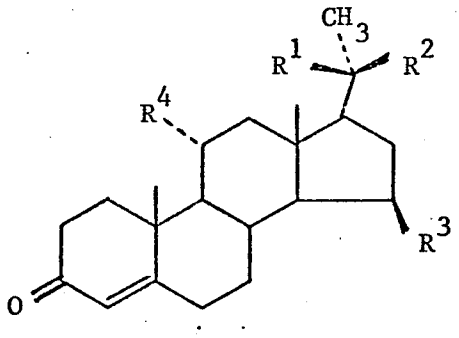
Fig. 1. The interaction and metabolism of [6β-^{2}H] testosterone with rat liver microsomes[42].

The mechanism of the C-21 hydroxylation of progesterone has been studied recently[44] by the use of progesterone derivatives and of C-21 labelled progesterones. To investigate activation by enzymic enolization, the microbial hydroxylation of progesterone (pregn-4-ene-3,20-dione) (VIIIa) to give 11-deoxycorticosterone(21-hydroxypregn-4-ene-3,20-dione) (VIIIb) has been examined. It has been established previously[45] that progesterone, 11-ketoprogesterone, and 19-norprogesterone were hydroxylated at C-21 by Aspergillus niger ATTCC 9142. However, when 20α- and 20β-hydroxypregn-4-ene-3-one (IXa and IXb, respectively) were incubated with A. niger, in neither case was C-21 hydroxylation observed but C-15β-hydroxylated (IXc and IXd) and C-11α, 15β dihydroxylated (IXd and IXf) products were formed in minor amount. It was also found that removal of oxygen from C-20 lead to a loss of hydroxylase activity in A.niger, i.e., incubation of pregn-4-ene-3-one (Xa), the 20-methylene derivative (Xb), and the 20-thiosteroid (Xc) produced no conversion products. Therefore, a C-20 carbonyl is essential for C-21 hydroxylation, and the possibility of the involvement of this group, in the C-20, 21 enol form, in the C-21 hydroxylation reaction was investigated by the use of progesterone, fully or partially labelled with deuterium at C-21. The magnitude of the observed deuterium isotope effect (K_H/K_D = 1.25), however, eliminates a mechanism of C-21 hydroxylation in which enolization of the C-20 carbonyl, with a rate determining loss of hydrogen from C-21, is the slow step. The effect is consistent with a mechanism of C-21 hydroxylation involving rate determining electrophilic insertion into a C-H or C-D bond. It was concluded that C-21 hydroxylation of progesterone is a reaction directly analogous in mechanism to hydroxylation at saturated carbon not adjacent or vinylogous to carbonyl and not one in which the substrate is activated to electrophilic attack by enolization, contrary to Δ^4-3-ketosteroids.



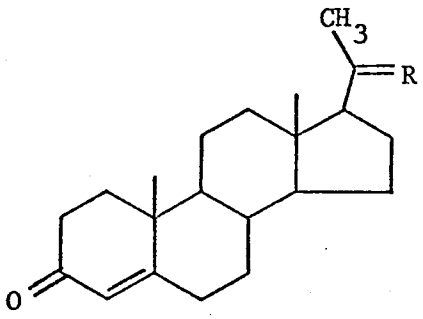
VIII

a. $R^1=R^2=R^3=H$
b. $R^1=R^2=H$; $R^3=OH$



IX

a. $R^1=R^3=R^4=H$; $R^2=OH$
b. $R^1=OH$; $R^2=R^3=R^4=H$
c. $R^1=R^4=H$; $R^2=R^3=OH$
d. $R^1=H$; $R^2=R^3=R^4=OH$
e. $R^1=R^3=OH$; $R^2=R^4=H$
f. $R^1=R^3=R^4=OH$; $R^2=H$



X

a. $R=H_2$
b. $R=CH_2$
c. R=S

ii) Conjugation Reactions

a) Glucuronic acid conjugation

Conjugation with glucuronic acid is a common phenomenon in the metabolism of foreign compounds in mammalian species. The mechanism of glucuronide formation involves the reaction of the compounds with D-glucuronic acid[46,47]. For the condensation to proceed, glucuronic acid is activated by biosynthesis of uridine diphosphate glucuronic acid. The synthetic sequence is initiated by the formation of glucose-1-phosphate, as shown in Scheme II. The formation of uridine diphosphate glucuronic acid from uridine diphosphate glucose is under the mediation of a dehydrogenase present in the supernatant fraction of liver preparations.

Scheme II

$$\text{Glucose-1-phosphate} + \text{UTP} \xrightarrow{\text{pyrophosphorylase}} \text{UDPG} + \text{pyrophosphate}$$

$$\text{UDPG} + 2\text{NAD}^+ + H_2O \xrightarrow{\text{UDPG dehydrogenase}} \text{UDPGA} + 2\text{NADH} + 2H^+$$

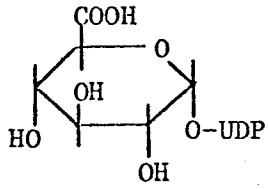
where UTP = uridine triphosphate

UDPG = uridine diphosphate-β-D-glucose

UDPGA = uridine diphosphate glucuronic acid

The condensation of UDPGA with the compound undergoing conjugation (RZH) takes place upon catalysis of the reaction by a solubilized microsomal enzyme[48], glucuronyl transferase, which is found primarily in the liver. Glucuronyl transferase is also present in other body tissues. The reaction proceeds according to Scheme III. The C-1 atom of glucuronic acid is present in the α-configuration in UDPGA but appears in the β-configuration in the conjugates formed.

Scheme III



+ RZH $\xrightarrow{\text{glucuronyl transferase}}$ RZ-glucuronic acid + UDP

where Z = O, CO, NH, or S.

Glucuronic acid forms conjugates with the hydroxyl groups of primary, secondary or tertiary aliphatic compounds, phenols, enolic compounds, and hydroxylamines. The carboxyl group of straight-chain aliphatic, heterocyclic, carbocyclic, and arylalkyl acids form such conjugates. Aromatic amines, carboxyamides, and sulfonamides have also been reported as reacting with glucuronic acid[49].

Alcohols and phenols tend to form "ether-type" glucuronides. Many aromatic and aliphatic carboxylic compounds form "ester-type" glucuronides. Aromatic amines form N-glucuronides enzymatically through glucuronyl transferase, but some compounds apparently form these conjugates by non-enzymatic reactions[50].

Isselbacher[51] has demonstrated that the steroid glucuronide is formed through the active glucuronide donor, UDPG. The formation of the conjugate is catalyzed by a glucuronyl transferase present in liver microsomes. The reaction involves the transfer of UDPGA to the steroid alcohol, and UDP is liberated.

The principal point of conjugation in steroid molecules is the hydroxyl group attached to C-3. However, if formation of a conjugate at this position is not possible and there are other hydroxyl groups present in the molecule, these hydroxyl groups may become attached to glucuronic or sulfuric acid to give rise to steroid conjugates[1].

Conjugation of drugs with glucuronic acid produces several characteristic changes in the physical properties of the substance. The products become more water-soluble than the parent drugs because of the large hydrophilic carbohydrate moiety, and thereby the partition ratio between a lipid and an aqueous solvent is considerably reduced. At the same time, the glucuronides usually are stronger acids than the parent drugs, and thus are more ionized at physiological pH values. Such compounds are less likely to penetrate membranes than the parent drugs, are poorly reabsorbed by the kidney tubules, and are more readily excreted[47]. It is rare that a drug forms a double conjugate, since one such adduct already increases the likelihood for excretion. A few doubly conjugated drugs have been isolated, however.

b) Sulfate conjugation

This form of conjugation is quantitatively less important than those involving glucuronic acid, probably because the body pool of sulfate is relatively small. To form a sulfate derivative of a drug, sulfate must first be activated by a series of reactions involving ATP. Sulfate is converted first to adenosine-5'-phosphosulfate (APS) and then to 3'-phosphoadenosine-5'-phosphosulfate(PAPS). PAPS has been identified as the active sulfate which transfers the sulfate to a drug acceptor. These reactions, which take place in the soluble fraction of cells, are shown in Scheme IV.

Scheme IV

$$SO_4^{--} + ATP \xrightarrow{\text{ATP-sulfurylase}} \text{adenosine-5'-phosphosulfate(APS)} + \text{pyrophosphate}$$

$$APS + ATP \xrightarrow{\text{APS-phosphokinase}} \text{3'-phosphoadenosine-5'-phosphosulfate (PAPS)} + ADP$$

$$PAPS + RZH \xrightarrow{\text{sulfokinase}} R\text{-}Z\text{-}SO_3H + \text{3'-phosphoadenosine-5'-phosphate (PAP)}$$

where Z is O or NH.

Several distinct sulfokinases (or sulfotransferases) have been described, and these enzymes exhibit considerable specificity[52]. The enzyme which transfers sulfate to phenol, for example, is found in the soluble fraction of liver, kidney and intestine; enzymes functioning in sulfate conjugation of certain steroids act in the liver only.

Primarily aliphatic alcohols and phenols react to form "ethereal sulfates"[49,53]. The "N-sulfates", or sulfamates, have been reported mainly for rat, rabbit and guinea pig; conjugation takes place in the soluble fraction of liver[54,55]. Traces of the N^4-sulfate have been recovered as a metabolic product of sulfanilamide, and aniline and related amines are also known to form N-sulfates. The formation of S-sulfates (thiosulfates) has not been definitely established.

Schneider and Lewbart[56] demonstrated steroid sulfate-synthesizing enzymes in a microsome-free supernatant fluid prepared from rabbit liver homogenate to which were added ATP, Mg^{++}, and sulfate. According to Gregory and Nose[57], there are at least two separate enzyme systems present in the soluble fraction of rabbit liver extracts. One system is active for estrogens and the other with 3β-hydroxy steroids. The 3α-hydroxy steroids do not form sulfates in this system.

c) Metabolism of alicyclic hydrocarbons

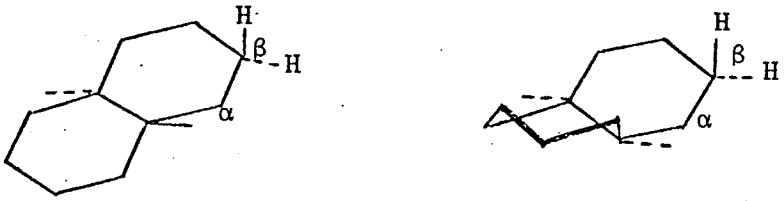
In a series of studies, Elliot et al. investigated the metabolic transformation in vivo of such simple alicyclic hydrocarbons as cyclohexane[10]

methylcyclohexane[58], and decalin[11]. The main products were secondary alcohols having the hydroxyl groups in the equatorial, thermodynamically more favorable, conformation.

With cyclohexane, the main alcohol produced was cyclohexanol, and with methylcyclohexane trans-4-methylcyclohexanol, together with lesser amounts of cis-3-and trans-3-methylcyclohexanols, hydroxylation occurring to the greatest extend at the carbon atom furthest away from the methyl group. The metabolism of cis- and trans-decalin in the rabbit gave racemic secondary alcohols; hydroxylation occurred specifically at the β*-position, yielding mainly (±)-cis-cis-2-decalol or (±)-trans-cis-2-decalol, respectively, which have the hydroxyl group in the equatorial conformation. A mechanism was proposed in order to explain why racemic alcohols, rather than the more typical optically active products of enzymic reaction, were obtained. In order that a racemic decalol be obtained, (+)- and (-)- alcohols must have been produced in equal amounts. Although trans-decalin, for example, is a symmetrical molecule (Fig. 2), when it becomes attached to the hydroxylating enzyme surface the spatial requirements of this attachment are such that it has to be considered as being composed of two enantiomers (Fig. 3). It was assumed that the orientation of the substrate is such as to expose a β-carbon atom to hydroxylation while protecting an α*-carbon atom. Steroid hydroxylases or closely allied enzymes were suggested to be responsible for the introduction of hydroxyl groups into both cis- and trans-decalin.

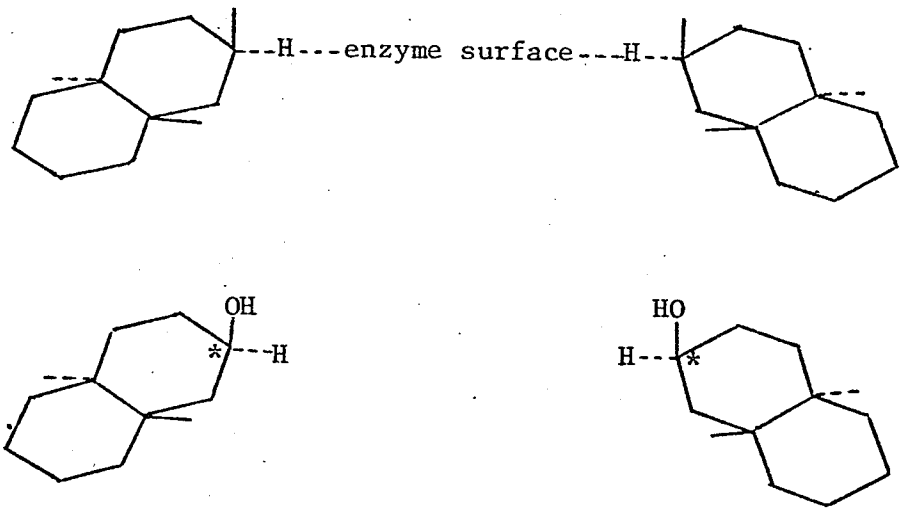
A similar pattern of metabolism was obtained with methyldecalins[59], with metabolites of either 6- or 7-hydroxy-2-methyldecalins. It was con-

* α- and β- refer to the position of carbon atom from ring junction; i.e., carbon atom one removed and carbon atom two removed from ring junction, respectively.



(a) (b)

Fig. 2. trans-Decalin (a) and cis-decalin (b).



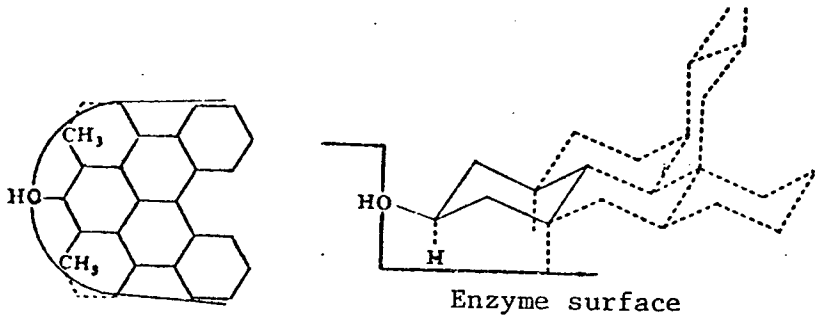
(+) or (-)-trans-cis-2-decalol (+) or (-)-trans-cis-2-decalol

Fig. 3. β-Hydroxylation of trans-decalin;
* denotes a new asymmetric C atom.

cluded that same hydroxylase may be responsible for the hydroxylation of all the alicyclic compounds. The simpler, less space-filling molecules would have the possibility of presenting a number of equivalent or near-equivalent methylene groups at the hydroxylation site, giving an appearance of randomness to the hydroxylation process, whereas the larger, more demanding molecules have a more limited choice of orientation.

An analogous result was obtained in the metabolism of tricyclic hydrocarbon system. Metabolism in rabbits of several perhydroanthracenes[12] all gave racemic secondary alcohols, having the hydroxyl group at a β-methylene carbon with equatorial configuration. Similarly, the ketone, cis-cisoid-trans-perhydroanthracen-9-one was metabolized to give an equatorial hydroxyl group at sterically unhindered β-carbon away from ring junction. Robertson and Dunstan suggested that the same hydroxylating enzyme system may be involved in these transformations and proposed a three dimensional framework for the enzyme active site. Suggestions were made that there was limited space in the vicinity of the active center of the enzyme for the accommodation of substrates, thus giving β-hydroxylated, rather than α-hydroxylated metabolites. The active center is located in a symmetrical depression in the enzyme surface, the sides of the depression acting as a lateral barrier. For simplicity, the depression is visualized as being cone-shaped as shown in Fig. 4(a). To account for the favoured equatorial configuration of the hydroxyl substituent, the substrate molecules must be so arranged that the equatorial C-H bond is directed to the hydroxylating site while at the same time the axial C-H bond is protected (Fig. 4(b)). Since the sterochemistry of the bridgehead does not appear to affect the site of hydroxylation, i.e., cis and trans isomers are both hydroxylated at a β-carbon, the active site need only be large enough to accommodate and anchor a cyclohexane molecule or moiety. The remainder of

the molecule may be attached to non-specific sites.



(a) (b)

Fig. 4. Suggested frame work for the β-hydroxylation of alicyclic hydrocarbons; (a) surface view, (b) profile.

Stereochemical aspects of the metabolism of the isomeric methylcyclohexanols and methylcyclohexanones were studied by Elliot et al.[60] The seven isomeric optically inactive forms of methylcyclohexanol (i.e., 1-, and cis- and trans-2-, 3- and 4-) were excreted by rabbits mainly as glucuronides of the thermodynamically more stable forms of the alcohols. Though the thermodynamically more stable (±)-trans-2-, (±)-cis-3- and trans-4-methylcyclohexanol were excreted unchanged, the less stable (±)-cis-2-, (±)-trans-3- and cis-4-methylcyclohexanol were inverted, most probably via a ketone, to the corresponding more stable epimers.

The (±)-2-, (±)-3- and 4-methylcyclohexanones were reduced in the rabbit and excreted mainly as the glucuronides of the thermodynamically more stable forms of the corresponding methylcyclohexanols as shown in Fig. 5. From this work Elliot et al. have developed a simple and elegant theory for predicting the metabolites of methylcyclohexanones. The stereochemical metabolic differences were explained in terms of the conformations of the substrates and of Vennesland's hypothesis[61] of the role of NADH in dehydrogenation reactions. They have considered all possible conformations and orientations of the substrates towards the "A" face of the coenzyme (NADH or NAD). For a hydrogen transfer, no steric interaction should occur between the carboxamide group of the coenzyme (i.e., away from the

nitrogen atom). For some substrates, only a "face-to-face" orientation is possible; for others only a reverse-perpendicular orientation, while some substrates can have both orientations, thus yielding two products. No other orientation than these two can explain the products obtained. Fig. 6 shows (+)-3-methylcyclohexanone interacting with NADH in its two possible orientations. All products could be successfully predicted by this theory. It was also found that, for a reaction to be reversible, the metabolic pathway leading from compound A to compound B, and the pathway from B to A must have the same transition state. This is not the case for the two pairs (-)-cis-2-alcohol/(+)-2-ketone and (-)-trans-2-alcohol/(-)-2-ketone. Additional information can be obtained by further consideration of this theory. If we restrict our argument to the in vitro studies, where the perturbating factors arising from conjugation are absent, it is apparent that the same product stereoselectivity exists in the metabolism of the three 3- and 4- methylcyclohexanones, which all yield the di-equatorial and axial-equatorial alcohols in a ratio 2:1. These alcohols are produced by two competitive, reversible reactions whose transition states are the same (see Fig. 5) and which, therefore, are under complete thermodynamic control (i.e., the thermodynamic stability of the products directs the reaction). It therefore appears that, if all or almost all known cases of substrate stereoselective metabolism are under kinetic control (i.e., the energy of the transition state influences the rate of reaction), a distinction must be made for product stereoselectivity, which is by definition the result of competitive reactions. When these competitive reactions are irreversible, the product stereoselectivity can only be under kinetic control; but when the reactions are reversible, a thermodynamic control should be expected, as suggested by the above example[62].

The same mechanism has been assumed to be responsible for the stereo-

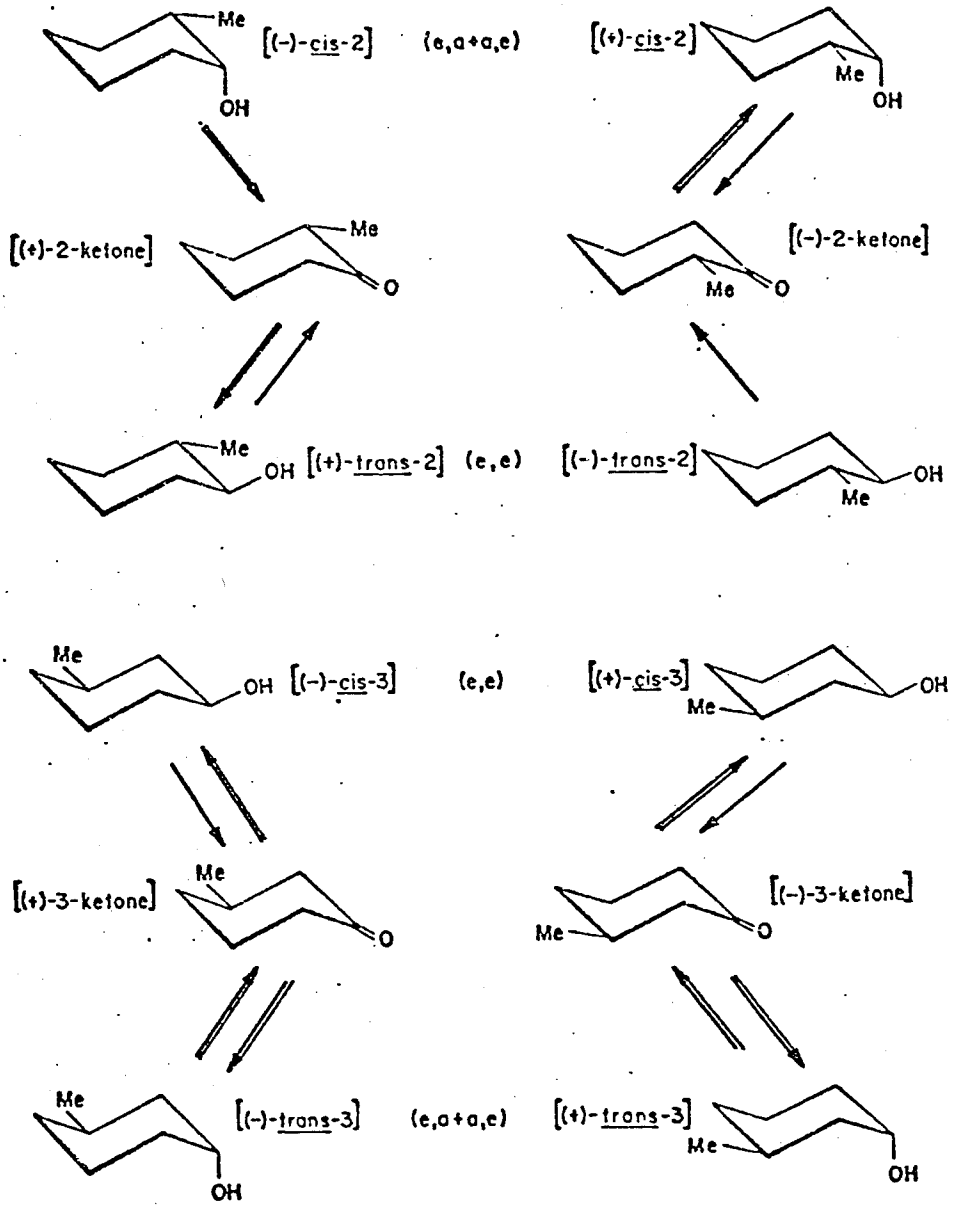


Fig. 5. Metabolism of isomeric methylcyclohexanones and methylcyclohexanols *in vivo* and *in vitro* (⟹) and *in vitro* only (⟶). The alcohols are excreted mainly as glucuronides.

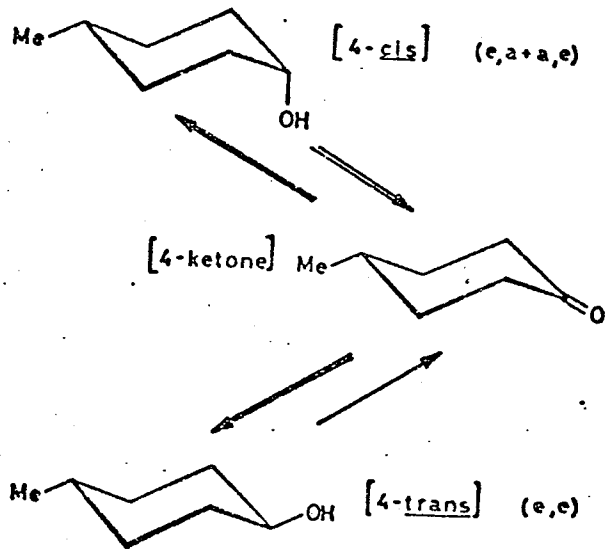


Fig.5. (continued)

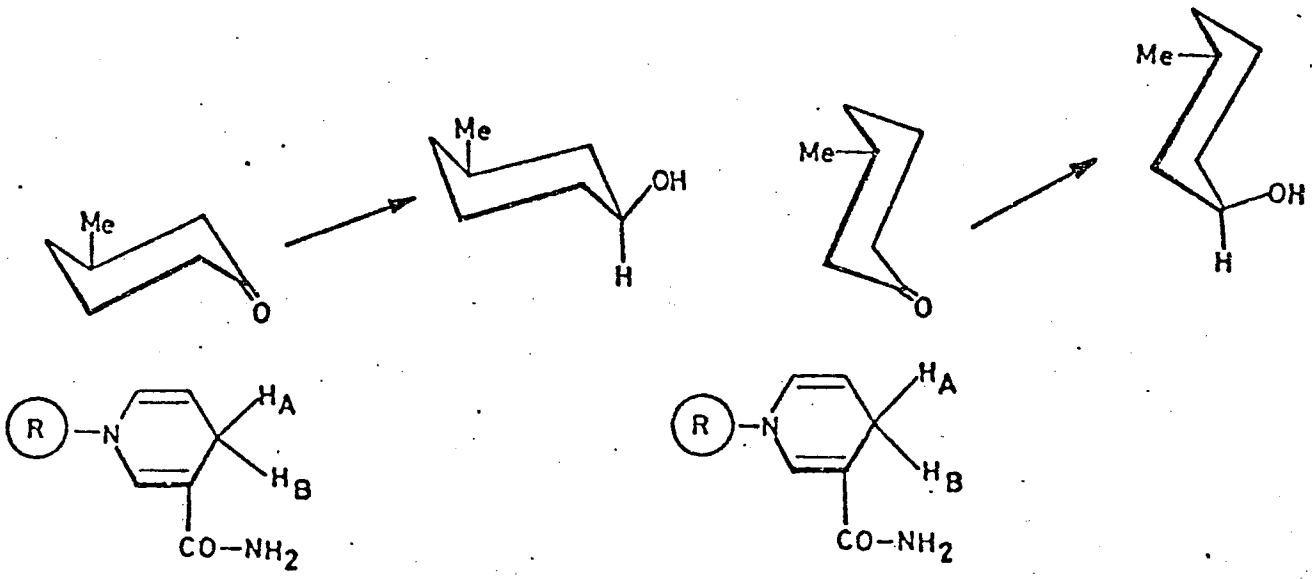


Fig.6. Interaction of (+)-3-methylcyclohexane with NADH. (+)-3-Methylcyclohexanone in a "face-to-face" and in a "reverse perpendicular" orientation toward "A" face of NADH, thus yielding (-)-cis-3- and (-)-trans-3-methylcyclohexanol, respectively.

specificity of the mammalian reduction of the isomeric decalones[63]. The predominant metabolic products of the racemic isomeric decalones in rabbits were equatorial alcohols that are optically active for the 1-decalols and racemic for the 2-decalols. Thus (±)-trans-1-decalone gave (+)-trans-trans-decalol and (+)-trans-cis-1-decalol; (±)-cis-1-decalone gave (-)-cis-cis-1-decalol and a small amount of (±)-cis-cis-1-decalol; (±)-cis-2-decalone gave (±)-cis-cis-2-decalol; (±)-trans-2-decalone gave (±)-trans-cis-2-decalol (see Table 2).

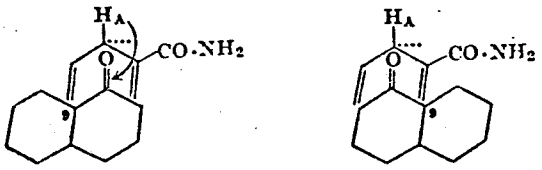
Table 2. Major metabolic transformations of the isomeric decalones (from Ref. (63)

Compound administered	Configuration of aglycone	Conformation of -OH group
(±)-trans-1-Decalone	(+)-trans-trans-1	eq
	(+)-trans-cis-1	ax
(±)-cis-1-Decalone	(-)-cis-cis-1 } (as racemate)	{ eq
	(+)-cis-cis-1	eq
(±)-cis-2-Decalone	(+)-cis-cis-2 } (as racemate)	{ eq
	(-)-cis-cis-2	eq
(±)-trans-2-Decalone	(+)-trans-cis-2 } (as racemate)	{ eq
	(-)-trans-trans-2	eq

By using the same hypothesis, models of the isomeric decalones, in their absolute configurations where established, can be placed in the transition state with an orientation that is either "face-to-face" (Fig. 7) or "head-to-tail"(Fig. 8) relative to the nicotinamide portion of the NADH molecule, also with its correct configuration (Fig. 9). It can be seen that, with trans-1-decalone (Fig. 7) there is steric conflict between its cyclohexane ring and the carboxyamide group of the nicotinamide moiety in one orientation (unfavorable alignment), but no conflict in the opposite orientation (favorable alignment). The formation of only (+)-trans-trans-1- and (+)-trans-cis-1-decalol is thus explicable. Considerably more

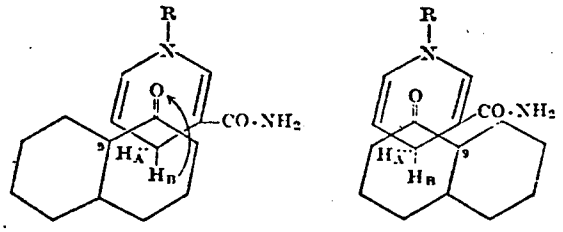
equatorial hydroxyl alcohol (trans-trans-) is produced than axial hydroxyl (trans-cis-) alcohol. Thus the reduction is not entirely dominated by favorable thermodynamic requirements.

In the case of trans-2-decalones, the cyclohexane rings are further from the ketone group than in the trans-1-decalones and hence steric hindrance is not a controlling factor in the reduction. Since only (±)-trans-cis-2-decalol (equatorial hydroxyl group) was formed, thermodynamic factors must have played a dominant part. Reduction of cis-1-decalones and cis-2-decalones have shown that hydrogen transfer does not occur in an unfavorable "face-to-face" alignments(b) where the non-reacting ring of the decalone lies between its reacting ring and the nicotinamide ring, giving only cis-cis-1- and cis-cis-2-decalols. (see Fig. 10).



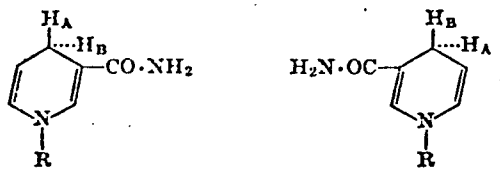
(a) (b)

Fig. 7. Favorable(a) and unfavorable(b) "face-to-face" alignments of trans-1-decalone with the nicotinamide moiety of NADH (partially overlaid).



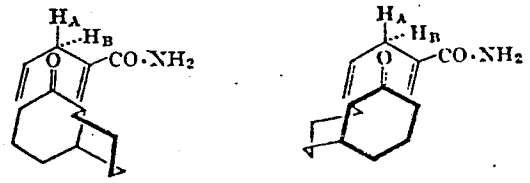
(a) (b)

Fig. 8. Favorable(a) and unfavorable(b) "head-to-tail" approach[64] of trans-1-decalone and the nicotinamide moiety of NADH. The configuration of the resulting alcohol is the same as that in the "face-to-face" approach (see Fig. 7).



(a) (b)

Fig. 9. Absolute configuration of the nicotinamide moiety of NADH, when attached (a) to an 'A' type enzyme (horse-liver alcohol dehydrogenase)[65] and (b) to a 'B' type enzyme (isolated from Curvularia falcuta)[64].



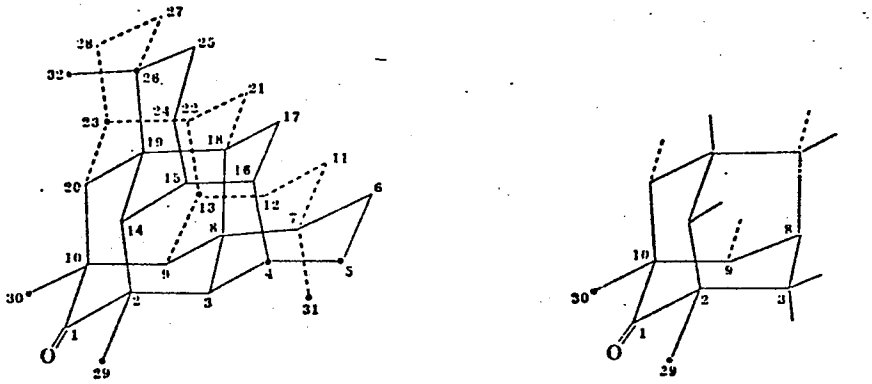
(a) (b)

Fig.10. Favorable(a) and unfavorable(b) "face-to-face" alignments of cis-1-decalone with the nicotinamide moiety of NADH. Where the non-reacting ring of the decalone lies between its reacting ring and the nicotinamide ring(b), hydrogen transfer does not occur.

The metabolic fate in rabbits of camphors and related compounds[66] indicated that only norcamphor and camphorquinone were reduced *in vitro* by NADH or NADPH in the presence of liver alcohol dehydrogenase, although all were reduced *in vivo*. This difference in behaviour *in vitro* was explained in terms of the stereospecific requirements for hydrogen transfer in the liver alcohol dehydrogenase - NADPH system. Thus, relatively unhindered oxo groups were readily reduced, whereas a bridge-head carbon or a ring methyl group in the α-position to the oxo group inhibited reduction. *In vitro* studies with the relatively non-specific oxidoreductase liver alcohol dehydrogenase and NADH have shown that where reaction occurred it

was found that the substrates did not occupy the 'forbidden regions' defined by the diamond lattice-section hypothesis of Prelog[67], as modified by Graves et al.[68].

The theory requires, among other things, that when the proposed substrates are sited on the enzyme surface so that the carbonyl group assumes a specific orientation, the molecule fits into a diamond lattice (Figs 11a and b), whether or not it has the form of a cyclohexanone chair. Reduction occurs unless there is a group larger than hydrogen at C-10 in the lattice.



(a) (b)

Fig. 11 (a) Diamond lattice section[68];
(b) Simplified diamond lattice section.

However, failure of this enzyme system to reduce some of the camphors that had been reduced in vivo led to the conclusion that another enzyme was responsible for reduction of these substrates in vitro. The subsequent study of metabolism of hydroanthracenones in rabbits by Robertson and Dunstan[69] with three different enzyme systems (liver alcohol dehydrogenase, hydroxy steroid dehydrogenase, aromatic aldehyde-ketone reductase) have suggested that liver alcohol dehydrogenase and hydroxy steroid dehydrogenase are capable of reducing a β-located oxy group but not an α-oxo group, whereas oxo groups in either location can be reduced by aromatic

aldehyde-ketone reductase. Thus, the kidney may be of importance in the reduction in vivo of certain cyclic carbonyl compounds.

The review of metabolic studies of the tetracyclic perhydrophenanthrene (steroid) ring system reveals that enzymatic oxidation does occur on unactivated parts of the molecule, as in the mono-, di, and tricyclic hydrocarbons. Oxidation of the unsubstituted A-ring of 17β-hydroxy-17α-methyl-5α-androstane[8] occured in the 3-position. Here the thermodynamically less stable axial (3α) alcohol as well as the equatorial (3β) epimer was isolated after incubation of the steroid with rabbit liver homgenate. The introduction of oxygen function into A-ring of 17β-hydroxy-17α-methyl 5α-androstane with consequent formation of a carbonyl or hydroxyl substituent in the C-3 position greatly affects biological activity of the resulting molecules (see Fig. 12).

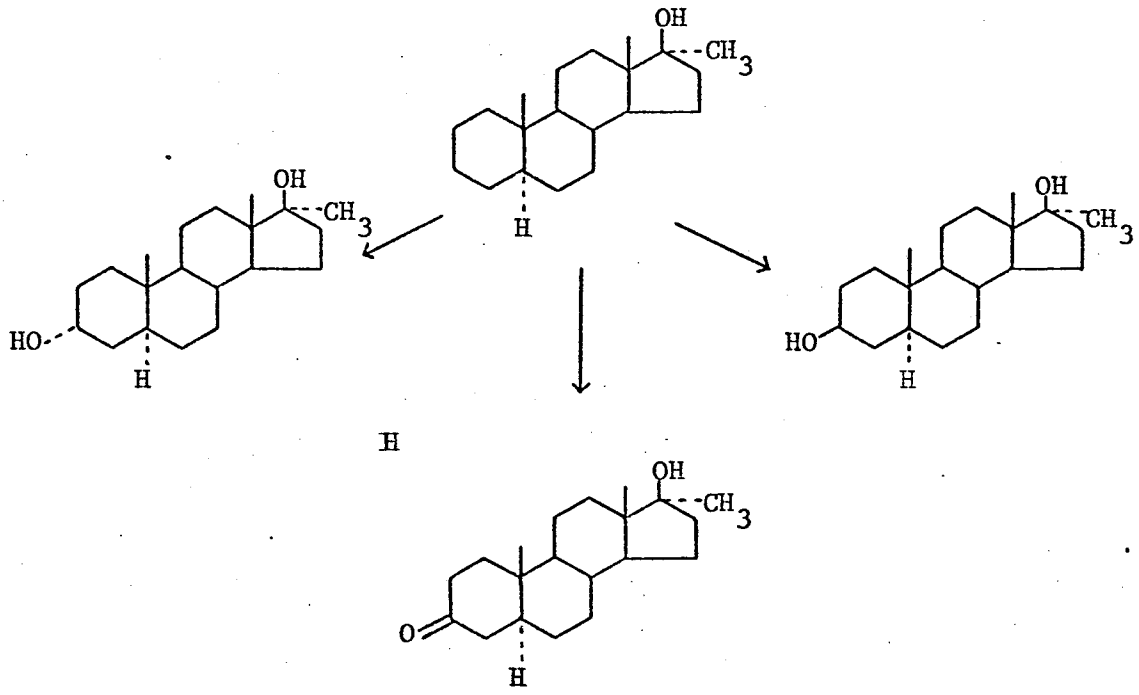


Fig. 12. Formation of 3-oxygenated metabolites of 17β-hydroxy-17α-methyl-5α-androstane by rabbit liver homogenate.

Therefore, the authors concluded that 17β-hydroxy-17α-methyl-5α-androstane owes its activity to its conversion *in vivo* to compounds oxygenated at the C-3.

Metabolism of 5-androsten-17-one (3-deoxydehydroepiandrosterone) by rabbits *in vivo* gave both axial and equatorial alcohols at C-3 and equatorial hydroxylation at C-2, both β to the ring junction[9]. The following six main metabolites (XI-XVI) were identified after oral administration of the steroid.

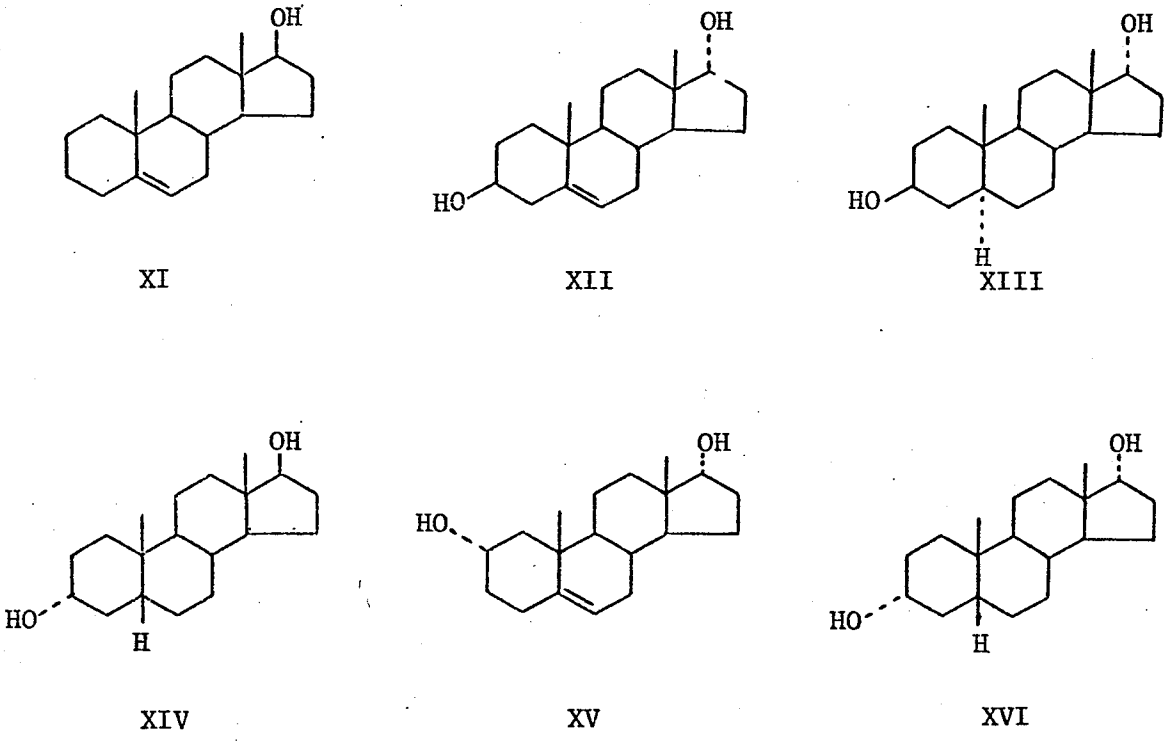


Fig. 13. Metabolites of 5-androsten-17-one by rabbits.

All the 3-oxygenated metabolities would be produced by the enzyme systems involving 3-dehydrogenase, $\Delta^5 \rightarrow \Delta^4$ isomerase and Δ^4-hydrogenase after the initial hydroxylation at C-3. It was also noted that 5-androsten-17-one underwent hydroxylation at C-2 without accompanying any disturbance at C-3 and the Δ^5-double bond, implying that the presence of an oxygen

function at C-3 is not necessarily prerequisite for C-2 hydroxylation. As for reduction of the 17-oxo group the administered steroid was principally metabolized to the 17α-hydroxylic compounds with exception of 3-deoxy- and 5β-steroids. This metabolic pattern appeared to be similar to that of 3-deoxy-estrone[70] and 17α-ethyl-estr-4-en-17β-ol to 17α-ethyl-19-nor-testosterone[71]. From these studies it seems very likely that 3-oxygenated compounds are the major metabolites formed from 3-deoxysteroids, and steroids having an unsubstituted A ring may owe their activity to their conversion *in vivo* to compounds oxygenated at C-3.

Comparatively little is known of the metabolism of the cyclopropane ring. The simplest of the alicyclic ring systems, cyclopropane gas, undergoes no metabolic change in the organism and is almost entirely eliminated by its route of entry, the lungs. Its ether derivatives, cyclopropylmethyl-, cyclopropylethyl-, and cyclopropylvinyl-ether are also metabolically inert and are excreted unchanged[72].

Wood and Reiser[73] and Chung[74] have indicated that the cyclopropyl ring is not metabolized *in vivo* in rats or rat liver mitochondria. A study of the metabolism of an anti-anxiety agent prazepam (XVII) in man[75] suggests that the cyclopropyl group is either removed unaltered by dealkylation to produce oxazepam (XIX), accounting for up to 46% of the dose or is eliminated unchanged as 3-hydroxy prazepam (XVIII) which represents up to 32% of the administered dose. Prazepam metabolism in dogs also proceeds via typical dealkylation and aromatic 3-hydroxylation[76].

Another example of removal of the cyclopropane ring was demonstrated by the metabolic study of 3,5-dichloro-N-cyclopropyl-4 (methylamino)-benzamide(XXI, Abbott-22700) in rat, dog and man[77]. Studies were carried out with both labelled (^{14}C in the amide carbon) and non-labelled drug. Based upon the rates of appearance and disappearance in blood, the probable

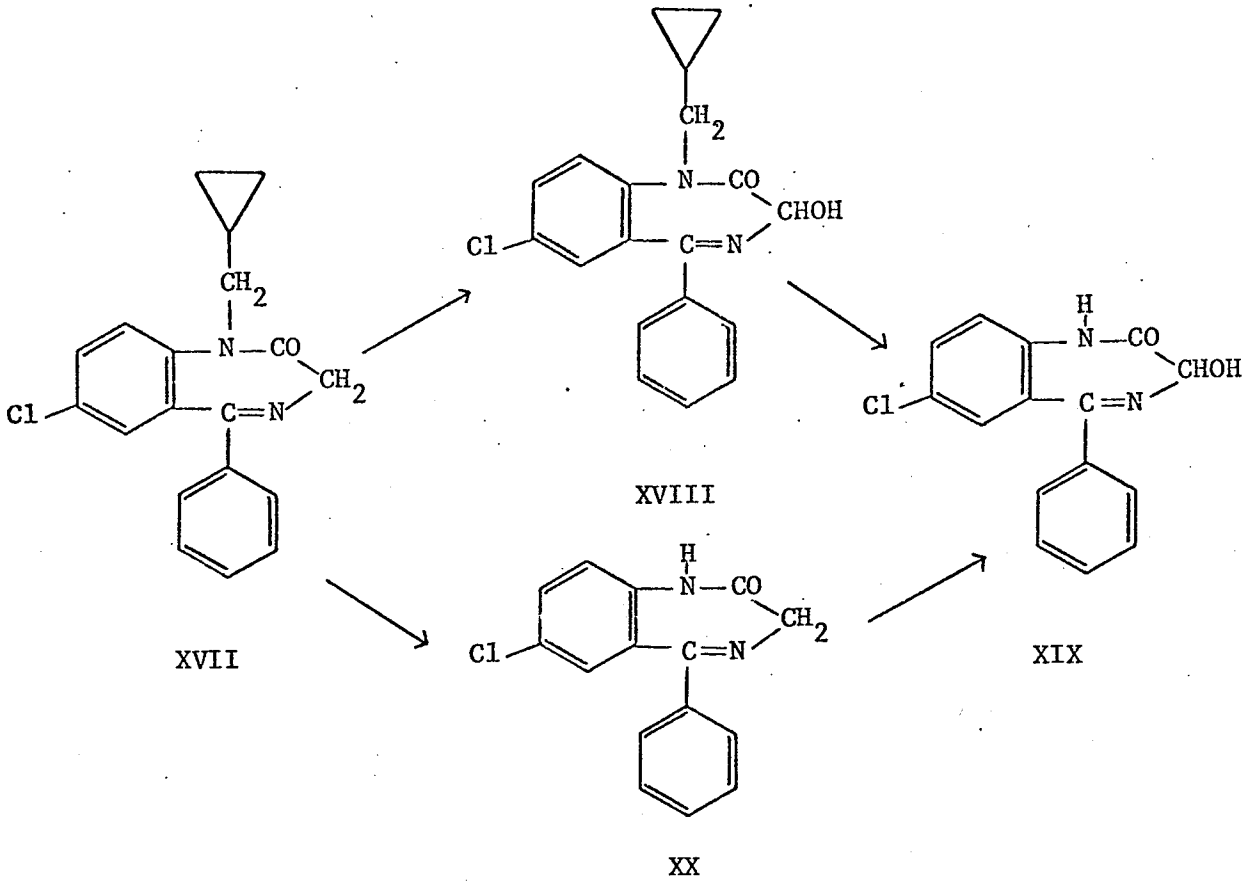


Fig. 14. Metabolism of Prazepam in man[75].

sequence of metabolism was found to be parent drug, 3,5-dichloro-N-cyclopropyl-4-methylamino-benzamide (Abbott-19931), 3,5-dichloro-4-methylamino-benzamide (Abbott-20163), probably through the intermediate (XXII) followed by the cyclopropane ring cleavage, and 3,5-dichloro-4-methylamino-benzoic acid (Abbott-20041).

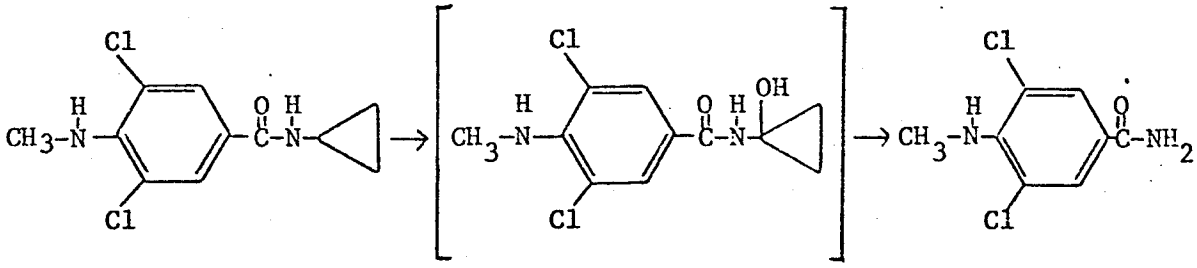


Fig. 15. Metabolic Pathway of 3,5-dichloro-N-cyclopropyl-4 (methylamino)-benzamide[77].

Abbott-19931, Abbott-20163 and free Abbott-20041 have also been identified in urine in addition to a large amount of conjugated Abbott-20041.

trans-2-Phenylcyclopropylamine (tranylcypromine) a potent inhibitor of MAO and an effective antidepressant in man, is metabolized extensively in the rat by pathways which include the cleavage of the cyclopropane ring[78]. The product identified in the urine was hippuric acid. Thus the cyclopropane ring is oxidized to carboxyl. It is unlikely that the first step is deamination by MAO since α-alkylamines are not oxidized readily by this enzyme[79]. Tranylcypromine itself is stable in water at 37° and pH 1-7.8 for several days, and this makes non-enzymic rearrangement with subsequent degradation unlikely. The enzyme that cleaves the cyclopropane ring is not known.

A number of cyclopropano-steroids have been studied as medicinal agents. However, the metabolic study of cyclopropano-steroids is limited to the antiandrogenic compound, 1α, 2α-cyclopropano-6-chloro-$\Delta^{4,6}$-pregnadiene-17α-ol-3,20-dione (Cyproterone). The principal metabolites isolated from the urine were identified as 1α,2α-cyclopropano-6-chloro-pregna-4,6-diene-17,20-diol-3-one (20α-OH-Cy) and 1α,2α-cyclopropano-6-chloro-androst-4,6-diene-3,17-dione (17-keto-Cy)[80]. The two metabolic changes therefore include the reduction of the 20-keto group and the removal of the side chain at C_{17}. The radioactive compounds detected in the urine showed no change in the A and B rings. It therefore follows that ring A reduction is markedly inhibited, in vivo, by the 1α,2α-cyclopropano-6-chloro-4,6-diene grouping. However, it is still not known if the reduction of ring A and of the 3-keto group is completely blocked.

Metabolism of synthetic steroids has been extensively reviewed by Fotherby and James[2] and Fotherby[81] and discussed in detail in several books[26,82,83].

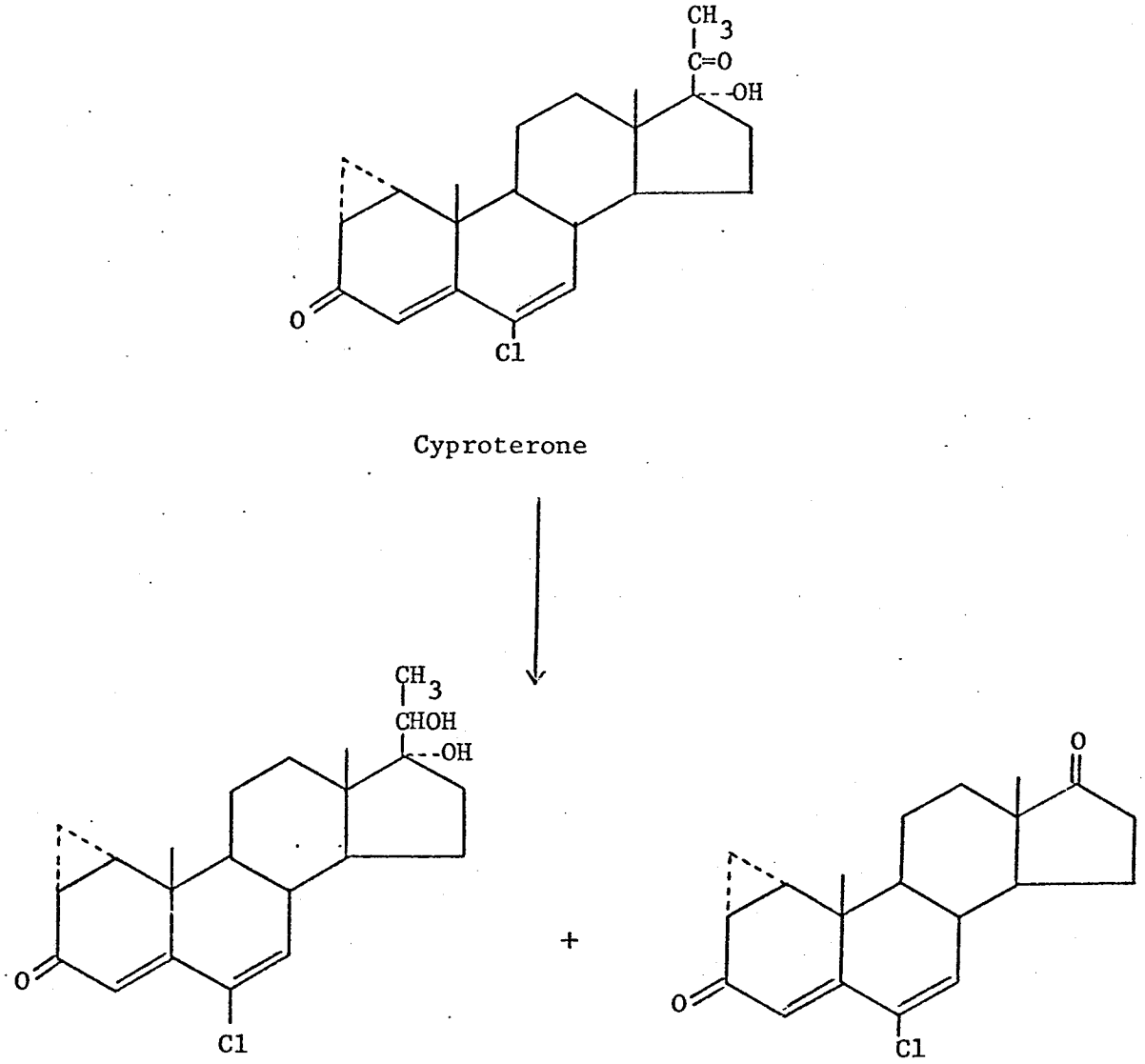


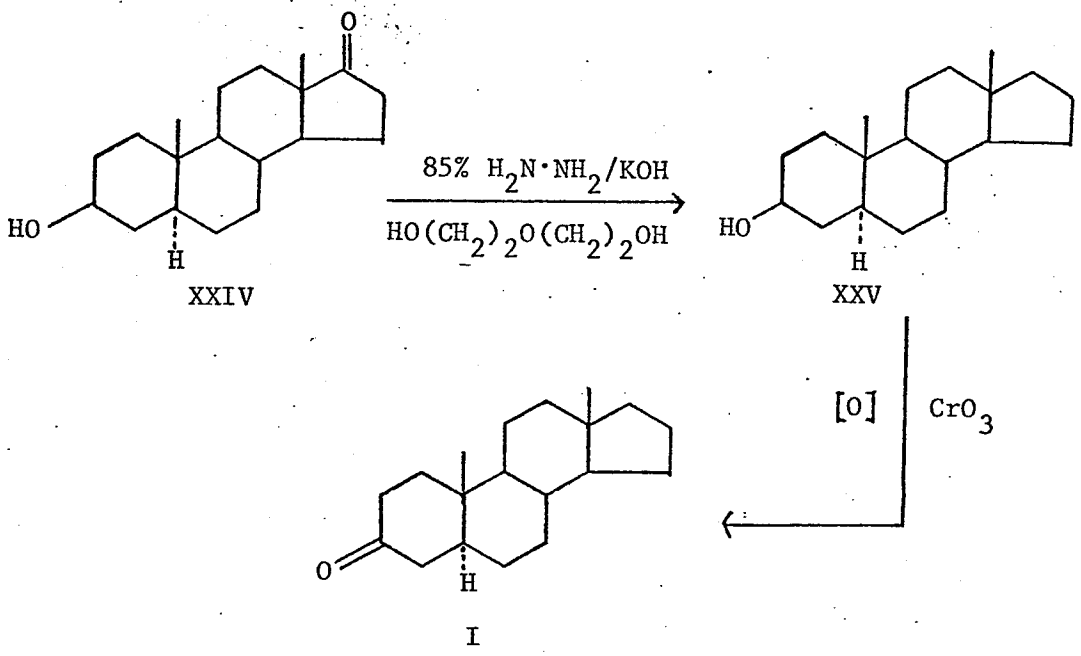
Fig. 16. Principal metabolites of Cyproterone isolated from urine[80].

III. RESULTS AND DISCUSSION

A. Metabolism of 5α-androstan-3-one

5α-androstan-3-one(I) was prepared by Wolff-Kishner reduction[84] of 3β-hydroxy-5α-androstan-17-one(XXIV) followed by Jones' oxidation[85] according to Scheme V.

Scheme V



XXIV XXV I

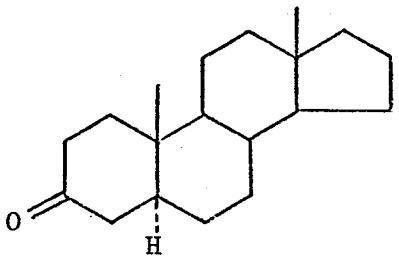
Isolation of the neutral steroidal fraction by ether extraction from the acid hydrolyzed urine of rabbits dosed with 5α-androstan-3-one(I) gave a seven fold increase in weight when compared with a blank experiment and accounted for 27% of the dose.

Thin-layer chromatography indicated the presence of oxygenated steroidal material, not present in the control urinary extracts, of higher polarity than either the initial 3-oxo-steroid or the 3α- and 3β- alcohol derivatives. Column chromatography showed that the total crude urinary extract consists of a minimum of 70% of material

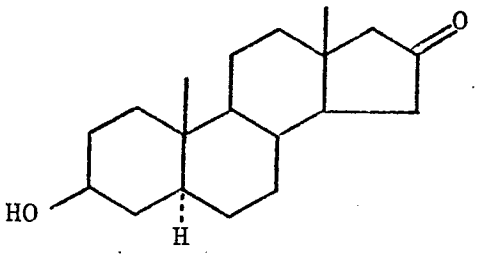
corresponding (by TLC) to the five substances isolated. GLC analysis showed that the five peaks corresponding to the substances isolated consist of 93% of the material eluted in the retention time range of these substances. The total crude urinary extract was separated into a non-ketonic and a ketonic fraction by treatment with Girard T-reagent[86]. The ketonic and non-ketonic fractions contained two and three substances, respectively, in the same proportions and with identical R_f and RRT as the unseparated material. A 7:4 ratio of non-ketonic to ketonic material was obtained. Only those substances showing R_f values in the range of diol and ketol androstane derivatives were identified.

Fig. 17 shows the gas chromatographic data of the total urinary extract dosed with 5α-androstan-3-one and ketonic and non-ketonic fractions of the total urine after Girard T separation are shown in Figs. 18 and 19. Three non-ketonic metabolites, 5α-androstan-3α, 16α-diol(XXVII), 3β, 17α-diol(XXVI) and 3β, 16α-diol(XXVIII) showed RRT of 0.34, 0.43 and 0.47, respectively. The relative retention times of 0.52 and 0.68 correspond to the 16-keto metabolites; i.e., 5α-androstan-3α-(XXX) and 3β-(XXIX) -16-one, respectively. The gas chromatography of these metabolites was examined by comparison with the authentic compounds except 5α-androstan-3α,16α-diol which was oxidized to the known compound, 5α-androstane-3,16-dione(XXXI) and was shown to have the identical retention time.

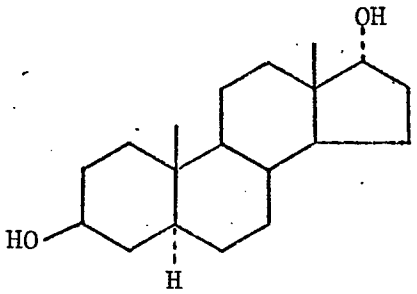
The non-ketonic fraction was further separated by column chromatography into three crystallinesubstances which showed only hydroxylic functional group absorption in their i.r. spectra and parent peaks in the mass spectra corresponding to dihydroxy compounds.



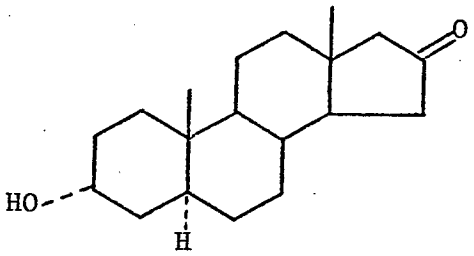
I



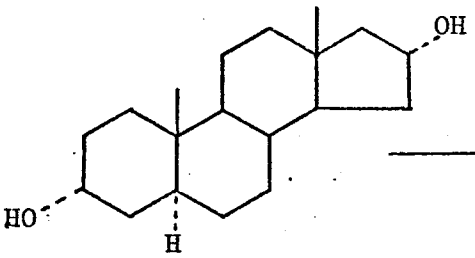
XXIX



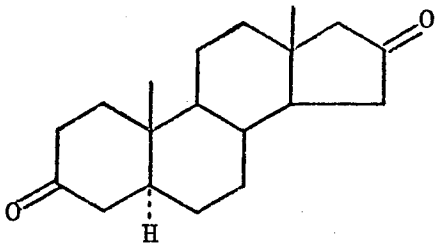
XXVI



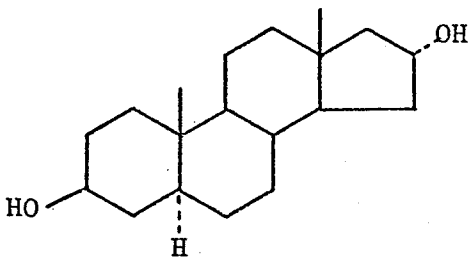
XXX



XXVII



XXXI



XXVIII

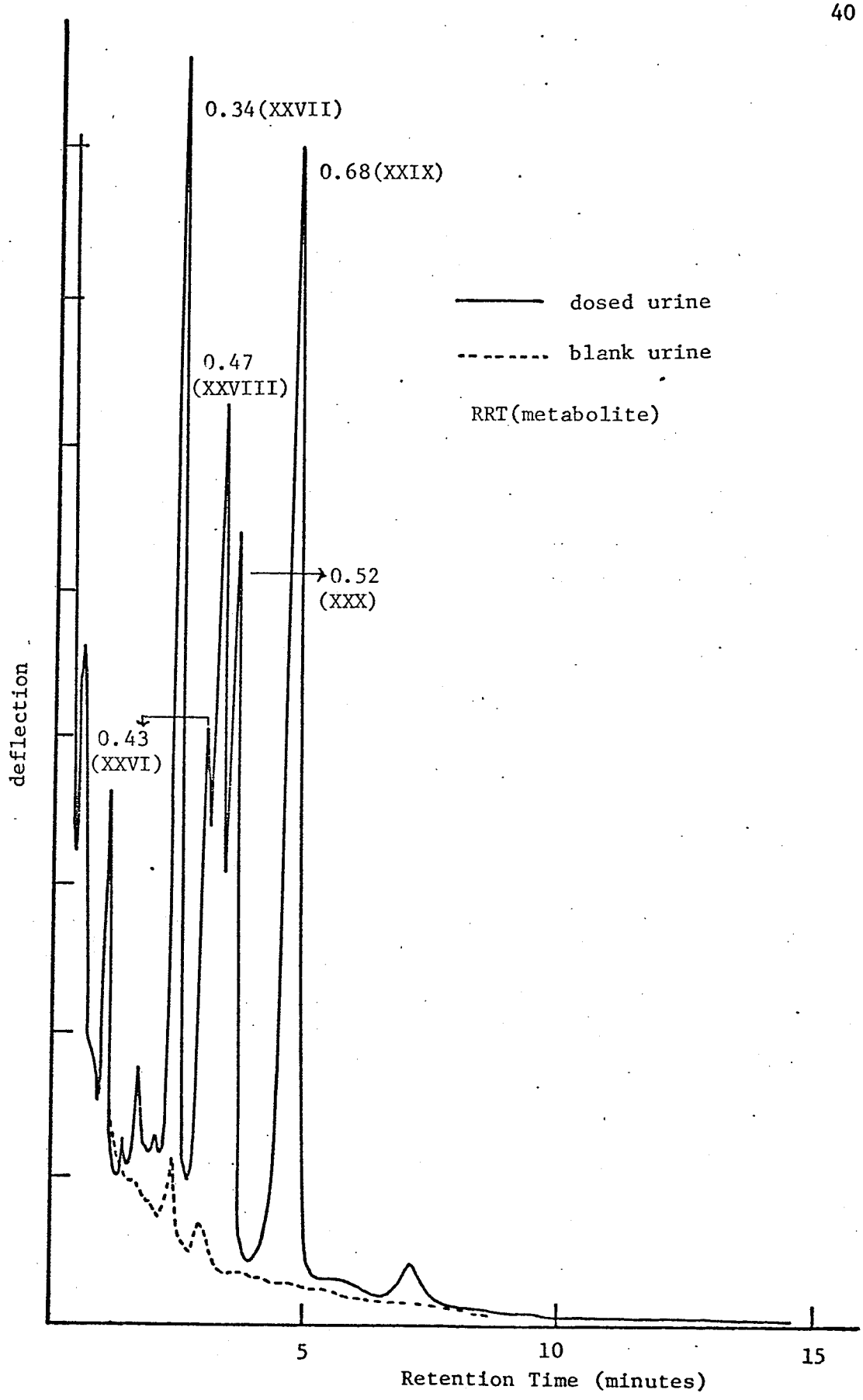


Fig. 17. GLC analysis of the total urinary extract, after oral administration of 5α-androstan-3-one (see Experimental).

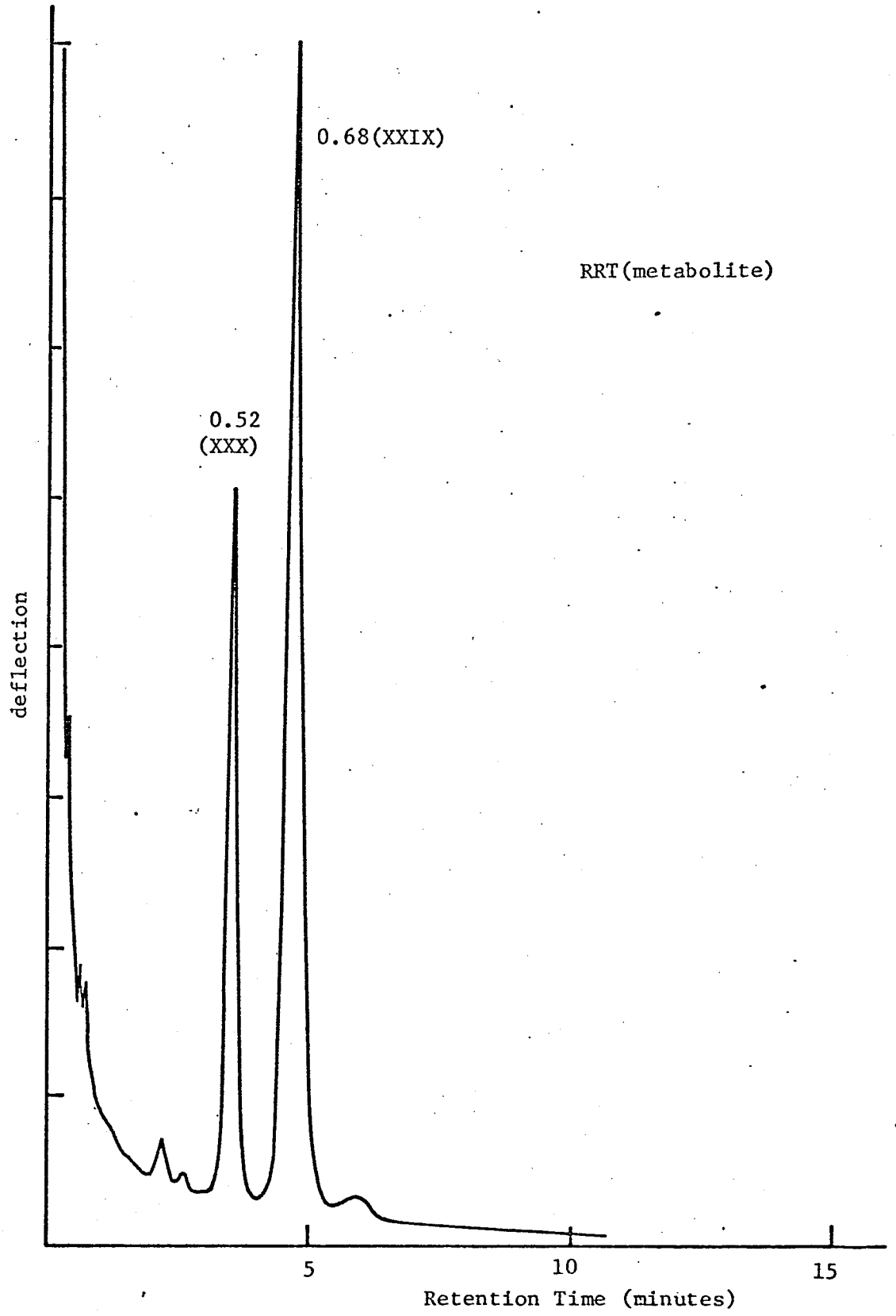


Fig. 18. GLC analysis of the ketonic fraction of the total urinary extract, after oral administration of I.

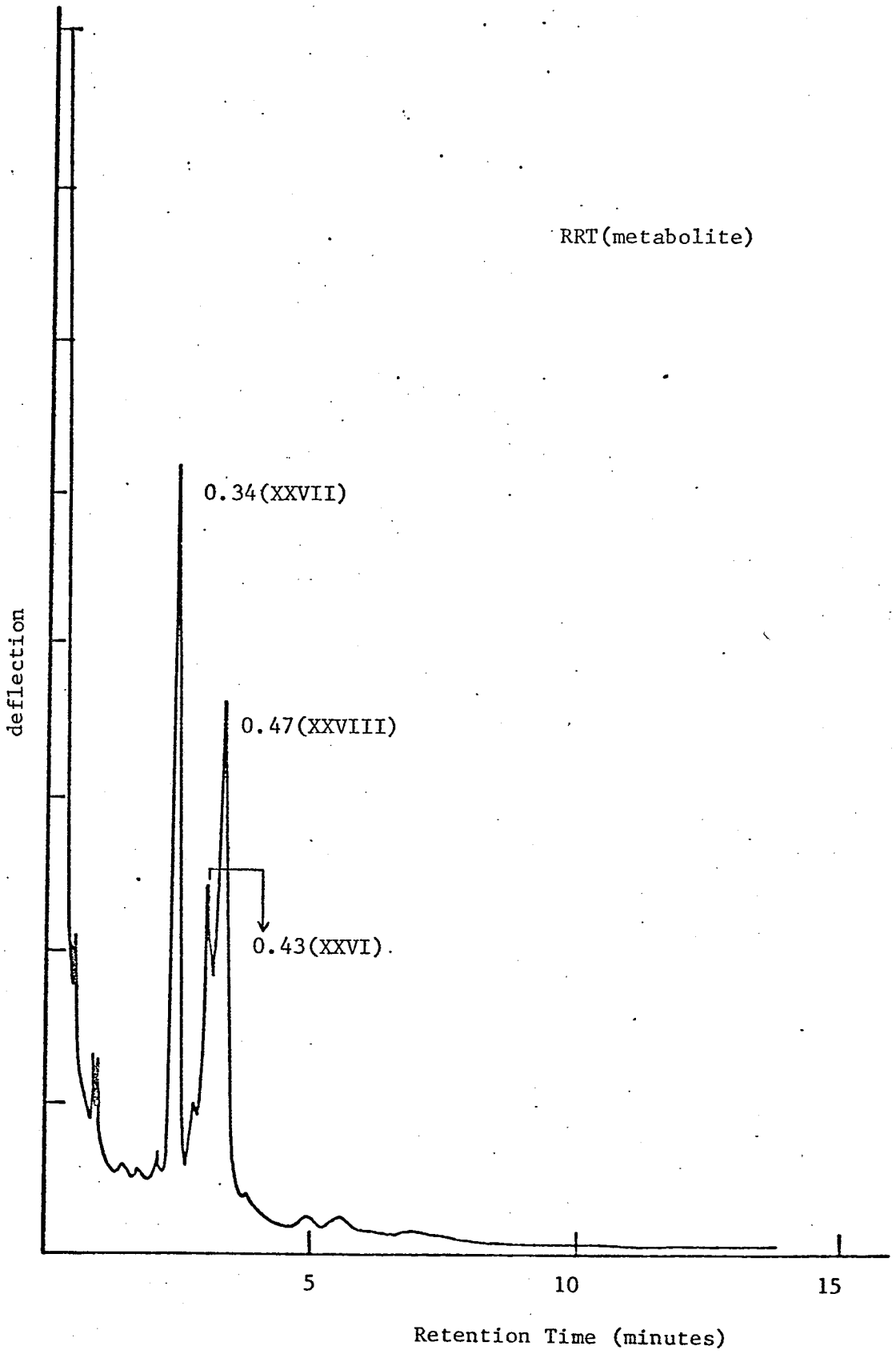


Fig. 19. GLC analysis of the non-ketonic fraction of the total urinary extract, after oral administration of I.

Computer averaged p.m.r. spectra in $CDCl_3$ of these poorly soluble compounds allowed assignment of the structures on the basis of the chemical shifts of the C-19 and C-18 methyl protons and the signals for the protons attached to the hydroxyl bearing atoms[87][91].

The assignments of the chemical shifts for the C-19 and C-18 methyl protons were based on the calculation according to the additivity rule of Zürcher[88] and works by Bridgeman et al[87]. For example, the chemical shifts of C-19 and C-18 methyl protons of 5α-androstane-3β,16α-diol(XXVIII) are calculated as follows;

	C-19 (p.p.m.)	C-18 (p.p.m.)
5α,14α-androstane	0.78	0.69
3β-OH	+0.03	+0.01
16α-OH	-0.01	-0.01
5α-androstane-3β,16α-diol	0.80	0.71

These calculated values (δ;0.80, C-19 methyl and 0.71, C-18 methyl protons) agree well with those obtained from p.m.r. spectra (δ: 0.82, C-19 methyl and 0.72, C-18 methyl protons). The difference between the chemical shifts of C-19 and C-18 methyl protons (0.10 p.p.m.) are also in good agreement with the calculated value (0.09 p.p.m.).

The stereochemistry of the hydroxyl groups were assigned from the characteristic chemical shifts and the splitting patterns[87] of the down-field protons and their band width at half-height[89]. Chemical shift differences between epimers are generally not great and in many cases both isomers are required for conclusive assignments to be made. The fact that diaxial vicinal coupling constants are much

greater than diequatorial or axial-equatorial coupling[90], also can be used to assign configurations to steroidal epimers. In general, when an electronegative substituent is attached to the steroid framework, the geminal proton is found downfield free from the saturated C-H region of the spectrum. However, such a proton will usually be adjacent to several others and will give rise to a broad band in which the number of closely spaced lines due to spin-spin coupling are not readily discernible. The width of such a band, measured at one-half its height (band width at half-height or $W_{\frac{1}{2}}$) will reflect the magnitude of the vicinal coupling constants. Thus, an axial proton, split by adjacent axial ($J \sim 9Hz$) and equatorial protons ($J \sim 2Hz$), should give rise to a much wider band than an equatorial proton split by adjacent axial ($J \sim 2Hz$) and equatorial protons ($J \sim 2Hz$). The utility of band width at half-height in assignments of stereochemistry in steroids has been demonstrated [89]. The band width at half-height due to equatorial proton coupling is 5-10Hz, while that for axial proton coupling is 15-30Hz. These correlations were applied to the assignments of the stereochemistry of the hydroxyl groups of all the metabolites. Identification of the known alcohols, 5α-androstane-3β, 17α-diol, and 5α-androstane-3β,16α-diol, was confirmed by direct comparison with authentic samples (TLC, GLC, m.p., mixed m.p., i.r., m.s., and p.m.r.) and oxidation to 5α-androstane-3, 16-dione. The third diol, on spectral evidence, was 5α-androstane-3α,16α-diol, which was further characterized by Jones oxidation[85] to the known 5α-androstane-3,16-dione.

The ketonic fraction could be separated into two major components by TLC and GLC. Chromatographic separation on an alumina column gave 3α- and 3β-hydroxy-5α-androstan-16-one which were identified on the

basis of their spectral properties and confirmed by direct comparison with authentic samples.

The comparison of the GLC trace with that from the analogous experiment where one-tenth of the dose (100 mg) was administered showed that there was some quantitative difference in the metabolites excreted. The most notable difference was the reversal in the relative intensities of the peaks of RRT 0.34 and 0.43; i.e., considerably larger amount of 5α-androstane-3α,16α-diol than 3β,17α-diol was excreted from the rabbit dosed with larger amount while the opposite phenomenon was observed from the metabolites of smaller dose. The relative percentages of GLC peak areas of five major metabolites from the two experiments are shown in Table 3.

Table 3. The relative percentages of GLC peak areas of metabolites of 5α-androstan-3-one.

	1g dose	100mg dose
5α-androstane-		
3α 16α-diol(XXVII)	22.6	8.3
3β 17α-diol(XXVI)	10.5	27.2
3β 16α-diol(XXVIII)	18.5	12.5
5α-androstane-		
3α-ol-16-one(XXX)	17.6	10.8
3β-ol-16-one(XXIX)	23.8	33.4
unidentified	7.0	0.8
Total crude urinary extract (blank subtracted)	100.0	100.0

As a result of a series of metabolic urinary excretion experiments in the rabbit on monocyclic, bicyclic and tricyclic hydrocarbons and some monooxygenated derivatives, Robertson and Dunstan[12], have suggested that a common hydroxylating enzyme system may be involved in the metabolism of alicyclic hydrocarbons. On this basis they have proposed a three dimensional framework for an enzyme active site for the hydroxylation of alicyclic compounds.

Cyclohexane was mainly converted to cyclohexanol and excreted as the glucuronide conjugate[10]. Metabolism of methylcyclohexane indicated that the position of hydroxylation, which occurred in all three possible ring positions, was dependent upon steric factors. The proportion of hydroxylation increased with distance from the methyl group[60]. Hydroxylation in the fused bicyclic series showed that cis- and trans-decalin[11] and cis- and trans-2-methyldecalin[59] yielded the thermodynanically more stable equatorial alcohol one carbon atom removed from the ring junction. A similar result was obtained in the hydroxylation of perhydroanthracenes[12]. Also, the ketone cis-trans-perhydroanthracen-9-one gave a compound with an equatorial hydroxyl group one carbon atom removed from the ring junction. However, the carbon atoms in this compound are non-equivalent because of the different ring fusion of the two outside rings to the central ring. The position of the hydroxyl group could not be determined either from proton magnetic resonance or mass spectra. On the basis of present experimental result on 5α-androstan-3-one, hydroxylation can be predicted to occur at C-3 or C-6, rather than C-2 or C-7 position, i.e., hydroxylation having occurred at the carbon atoms furthest removed from the initial oxygen function of cis-trans-perhydroanthracen-9-one.

Metabolic experiments on the monooxygenated steroid ring system have shown that enzymatic oxidation also occurs on unactivated hydrocarbon parts of the molecule usually at the less sterically hindered positions and removed from initial polar functions. Oxidation of the unsubstituted A-ring of 17β-hydroxy-17α-methyl-5α-androstane occurs in the 3-position[8]. Here direct hydroxylation yields the thermodynamically less stable axial (3α) alcohol as well as the equatorial (3β) epimer after incubation with rabbit liver homogenate. Metabolism of 5-androsten-17-one in the rabbit gave both axial and equatorial alcohols at carbon 3 and the equatorial alcohol at carbon 2, both attached to carbon atoms one atom removed from the A/B ring junction[9]. In the present work, oxidation of the D-ring of 5α-androstan-3-one in rabbits _in vivo_ had given the more stable alcohols in both the 16α- and 17α-positions. Substantially more oxidation has occurred at the 16-position compared with the more sterically hindered 17-position (7.5:1).

From molecular models it can be seen that oxidative attack on the steroid molecule has occurred in positions furthest removed from the initial oxygen function. In 17β-hydroxy-17α-methyl-5α-androstane, oxidation occurs in the 3-position which is the carbon atom furthest removed from the initial 17-oxygen function. 5-androsten-17-one is oxidized at the 2- and 3-positions remote from the 17-oxygen function. Similarly, the oxygenated derivatives isolated from metabolism of 5α-androstan-3-one show that oxidation has occurred at the 16- and 17-positions which are the atoms furthest removed from the 3-oxygen function. Oxidation at the position distant from the initial polar group is consistent with the suggested non-polar environment[33] of

the active site of the liver microsomal monooxygenase system. No evidence of substances monohydroxylated at other positions of the hydrocarbon skeleton was observed, showing a high degree of selectivity in the area of enzymatic attack for the urinary excreted steroids. The normal quantity of endogenous androstanes expected to be excreted in the urine over the collection period is less than 0.5% of the total steroidal material isolated[92].

Consideration of hydroxylation of the steroid nucleus within the steric requirements of the surface view of the framework proposed by Robertson and Dunstan[12] shows that only oxidation at carbons 3 and 16 can be accommodated. Exclusion of hydroxylation from the α-face by the profile view allows formation of the 3β- and 16β-alcohols. Direct 3α-hydroxylation which has been shown to occur with rabbit liver homogenate does not fit the proposed framework. The surface view of the active site excludes hydroxylation at carbon 2 to either the α or β face because carbons 12, 13, and 15-18 would lie outside the surface area. Similarly hydroxylation at carbon 17 is excluded by the atoms at carbon 1-5, 10, 11, and 19. It is possible that the 16α-alcohol is formed by initial 16β-hydroxylation and conversion via the 16-ketone (which is also isolated) to the 16α-alcohol. Therefore, it appears that either the steric requirements of the active site proposed by Robertson and Dunstan can be adapted further to fit the steroid nucleus or that a different oxygenation system more specific to the steroid structure is operative.

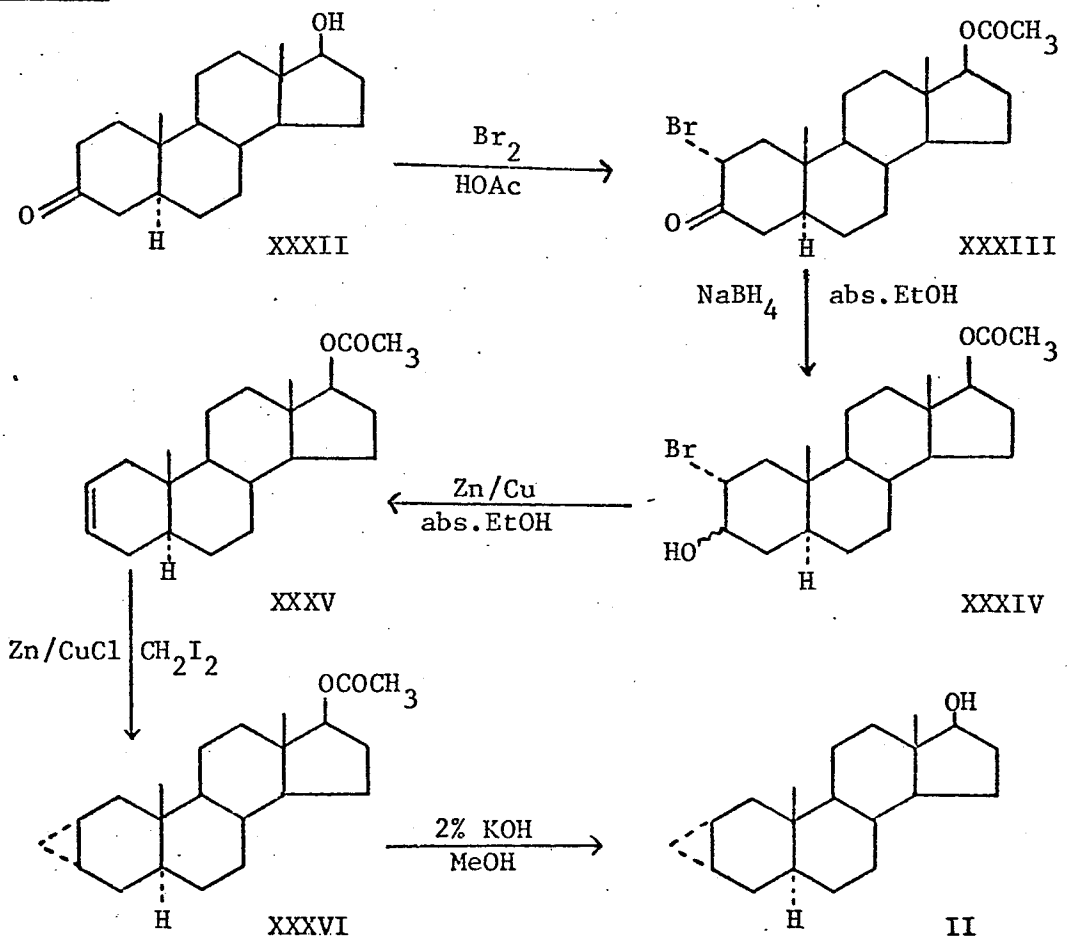
The involvement of intestinal microorganisms in the formation of the substances isolated has not been determined. However, experiments

in animals pretreated with neomycin in order to sterilize the gut showed no significant change in the nature or quantity of the metabolites formed. Further, considering the relatively large amount of conversion occurring, liver metabolism is the most probable source of these compounds. 16α-hydroxylation of steroids has been shown to occur in liver microsomal preparations from germ-free rats[93].

B. Metabolism of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane

17β-hydroxy-2α,3α-cyclopropano-5α-androstane(II) was prepared according to the Scheme VI.

Scheme VI



Isolation of the crude neutral steroid fraction from β-glucuronidase hydrolysis of the urine from rabbits orally dosed with 17β-hydroxy-2α, 3α-cyclopropano-5α-androstane gave a seven fold weight increase (35% of the dose) compared with a blank experiment. GLC of the silylated crude product showed the presence of seven peaks as shown in Fig. 20. Recrystallization of the crude product from methanol yielded the major product. Column chromatography of the residue yielded fractions from which five crystalline compounds including the major product could be separated accounting for 80% of the crude isolated material. These substances also accounted for 80% of the GLC peaks. These compounds were identified as 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), 2α,3α-cyclopropano-5α-androstane-6α,17β-diol (XLII) and 17β-hydroxy-3β-methyl-5α-androstan-2-one (XLVI) on spectral evidence.

The relative percentages (%) of the GLC peak areas of each metabolite are shown in Table 4 . The peak area of 2α,3α-cyclopropano-5α-androstane-6α,17β-diol (XLII) was estimated from the percentage of GLC peak areas of 2α,3α-cyclopropano-5α-androstane-6,17-dione in the total crude oxidation product. This value corresponded very well to that obtained from the GLC peak area when the total crude urinary extract was treated with t-butyldimethylsilylchloride (TBDMSiCl)* to separate XLII from XXXVII using identical amount of silylating reagent (25 μg of steroid/1 μl TBDMSiCl) and column conditions with those employed for the trimethylsilylation.

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* unpublished work by Westmore, J. B. and Quilliam, M., Department of Chemistry, University of Manitoba (1975).

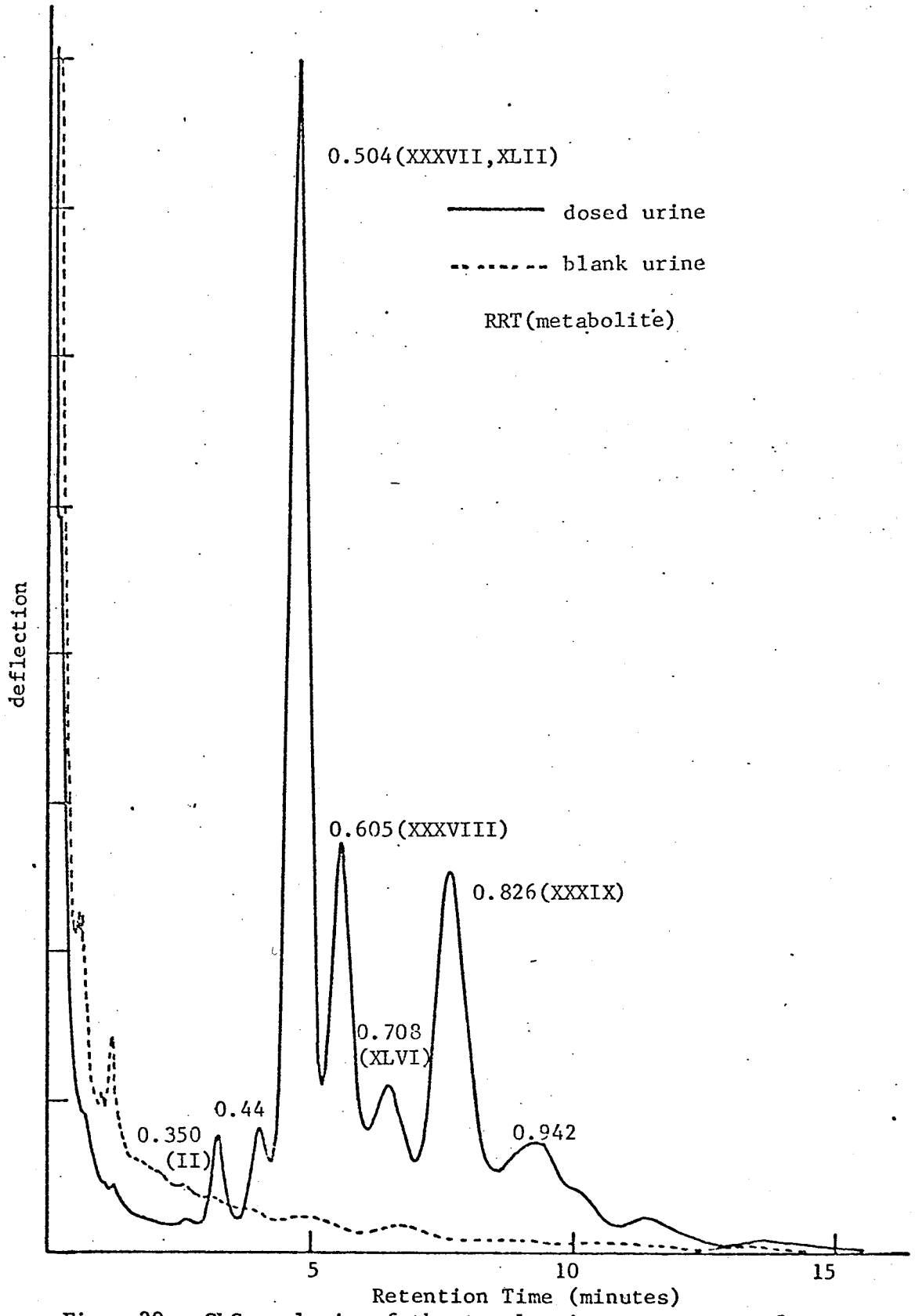


Fig. 20. GLC analysis of the total urinary extract, after oral administration of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (II)...(a).

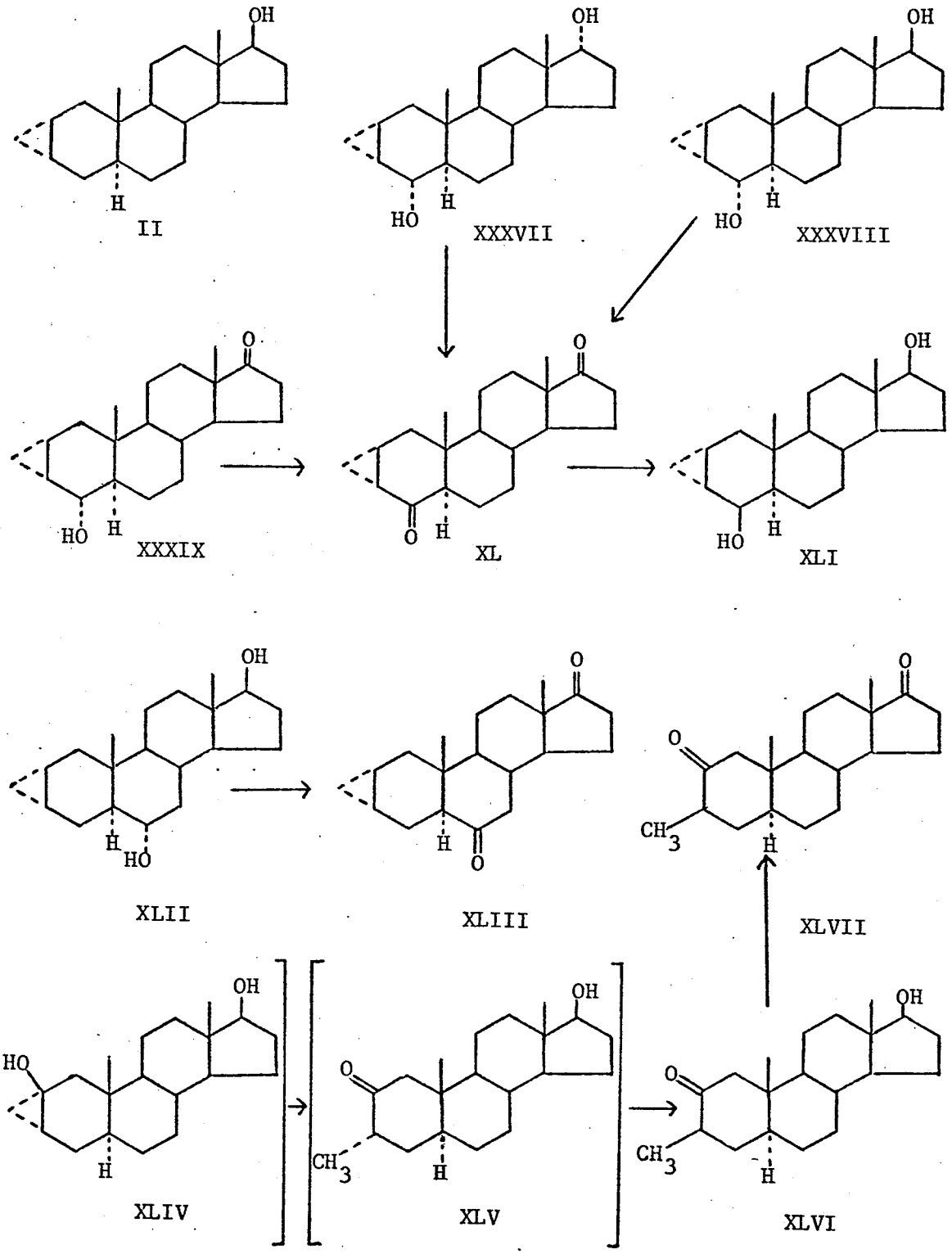

OH
H
II
OH
HO
H
XXXVII
OH
HO
H
XXXVIII
O
HO
H
XXXIX
O
O
H
XL
OH
HO
H
XLI
OH
H
HO
XLII
O
H
O
XLIII
O
O
CH3
H
XLVII
HO
H
XLIV
OH
O
CH3
H
XLV
OH
O
CH3
H
XLVI
OH

Table 4. The relative percentages of the GLC peak areas of the urinary metabolites of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane.

Metabolites	%
2α,3α-cyclopropano-5α-androstane-4α,17α-diol(XXXVII)	31.8
2α,3α-cyclopropano-5α-androstane-4α,17β-diol(XXXVIII)	12.9
4α-hydroxy-2α,3α-cyclopropano-5α-androstane-17-one(XXXIX)	20.5
2α,3α-cyclopropano-5α-androstane-6α,17β-diol (XLII)	7.9
17β-hydroxy-3β-methyl-5α-androstan-2-one(XLVI)	6.5
17β-hydroxy-2α,3α-cyclopropano-5α-androstane(II)	2.4
others	18.0
Total crude urinary extract	100.0

In particular signals associated with the C-18 and C-19 methyl groups and the chemical shifts and splitting patterns of the protons attached to the hydroxyl bearing carbon atoms in their p.m.r. spectra, together with the infrared carbonyl stretching vibrations and the p.m.r. and i.r. spectra of their oxidation products were employed[87].

The mass spectra of XXXVII and XXXVIII indicated the incorporation of one oxygen atom into the parent molecule (II). Compounds XXXVII, XXXVIII and XXXIX showed the presence of high field p.m.r. signals in agreement with the presence of a cyclopropane ring and a down field signal (3.84-6, q, J =4 and 6Hz) indicating the presence of a hydroxyl function in the same structural environment. The identical diketone formed from Jones oxidation[85] showed the presence of a five membered ring carbonyl absorption (1740 cm^{-1}) together with a second carbonyl

absorption (1683 cm^{-1}) in agreement with a ketone conjugated with a cyclopropane ring and cyclopropane C-H stretching in the i.r. spectrum. The chemical shift of the C-19 methyl group showed that the carbonyl group must be at C-4 excluding a carbonyl at C-1. Hydroxylation at C-1 can be further excluded since oxidation to the diketone (m.p. 223-4°) did not lead to the known 2α,3α-cyclopropano-5α-androstane-1,17-dione (m.p. 153-155°)[94]. The stereochemistry of the required hydroxyl group present at C-4 is shown to have the 4α-configuration by comparison of the C-19 chemical shifts in their p.m.r. spectra which differs significantly from the 4β-alcohol. Reduction of the diketone gave a diol which contained the cyclopropane ring as indicated by the i.r. and p.m.r. spectra. The p.m.r. spectrum was consistent with the expected formation of both a 17β-hydroxyl and a 4β-hydroxyl group. Metal hydride reduction of a carbonyl group conjugated with a cyclopropane ring has been shown to produce the alcohol with the stereochemistry formed by addition of hydride to the same side of the molecule as the cyclopropane ring[95]. Reduction of XL lead to two products on TLC, the less polar of which was slowly converted to the diol XLI. The intermediate compound showed the presence of the α-ketocyclopropane in the i.r. spectrum consistent with reduction of only the 17-carbonyl. The sterically hindered 4-ketone being reduced appreciably more slowly allowing selective reduction of the two ketones.

The presence of a downfield proton (3.72, d, J=6Hz) and a C-18 methyl group (0.63) in XXXVII and a proton (3.61, t, J=8Hz) and a C-18 methyl group (0.72) in XXXVIII in the p.m.r. spectra is

consistent with the presence of a 17α- and 17β-hydroxyl group in these substances respectively. Substance XXXIX showed an infrared carbonyl absorption and a p.m.r. signal at 0.84 ppm in agreement with a 17-ketone.

Jones' oxidation of XLII yielded a diketone XLIII having cyclopropyl and two carbonyl absorptions in the i.r. spectrum corresponding to a 17-ketone and a six-membered ring ketone. The chemical shifts of the C-18 and C-19 methyl groups of this ketone were consistent only with that of a carbonyl group at C-6. The p.m.r. spectrum of XLII was consistent with the presence of a cyclopropane ring and a 17β-hydroxyl group and showed a second downfield proton. The chemical shift and splitting pattern of this proton and the chemical shift of the C-19 methyl group establish the 6α-hydroxyl configuration in XLII.

The infrared spectrum of substance XLVI showed carbonyl absorption consistent with the presence of a six-membered ring ketone and no cyclopropane stretching vibration. The p.m.r. spectrum lacked the high field signal associated with the cyclopropane ring but showed a doublet centered at 0.995 p.p.m. in agreement with an α-methyl ketone. The presence of a downfield proton (3.63, t, J=8Hz) and a signal (0.72) was consistent with a 17β-hydroxyl group. This moiety is most probably formed by hydroxylation at either C-2 or C-3 followed by ring opening. The i.r., m.s., p.m.r. and chromatographic (TLC, GLC) properties of this substance were consistent with 17β-hydroxy-3β-methyl-5α-androstan-2-one(XLVI). The stereochemistry of the methyl group at C-3 is assigned the equatorial configuration on the basis of the known chemical shifts of similar compounds; 0.90 p.p.m.,

d, J=7Hz, 3β-methyl of 3β-methyl-5α-cholestan-2-one (cf. 1.20 p.p.m., d, J=8Hz, 3α-methyl of 3α-methyl-5α-cholestan-2-one)[96] and 1.00 p.p.m., d, J=7Hz, 2α-methyl of 17β-hydroxy-2α-methyl-5α-androstan-3-one. Spectral and TLC comparison with 17β-hydroxy-2α-methyl-5α-androstan-3-one (III) clearly showed that they were not identical.

GLC determination of the metabolites XXXVII, XXXVIII, XXXIX, XLII and XLVI accounted for approximately 80% of the seven peaks present and showed that the position of monohydroxylation had occurred at C-2, C-4 and C-6 in the order of C-4>> C-6>C-2. The stereochemistry of the initial hydroxylations at C-2, C-4 and C-6 is not established, since epimerization via the ketone may subsequently take place in vivo. However, initial hydroxylation at C-2 would most probably be from the β-face since α- attack would require formation of a highly strained trans C-2, C-3 ring junction[97].

A simplified picture of the isolated metabolites by eliminating epimeric hydroxyl isomers was obtained by comparing the GLC pattern of the Jones' oxidation product of the total crude urinary extract with a similarly treated control. In a separate experiment the crude extract was first treated with methanolic potassium hydroxide under conditions known to convert a cyclopropanol to an α-methyl ketone[98] prior to oxidation. An identical GLC trace with that of the untreated crude extract was obtained. The purpose of the base treatment was to convert any cyclopropanol metabolite to the corresponding carbonyl derivative and especially to demonstrate the presence of a 2 or 3-hydroxy-cyclopropane, if any, and to ensure its conversion to the more stable α-methyl ketone. GLC examination of the oxidation product (Fig. 21) showed the presence of peaks corresponding to 2α,3α-cyclopropano-

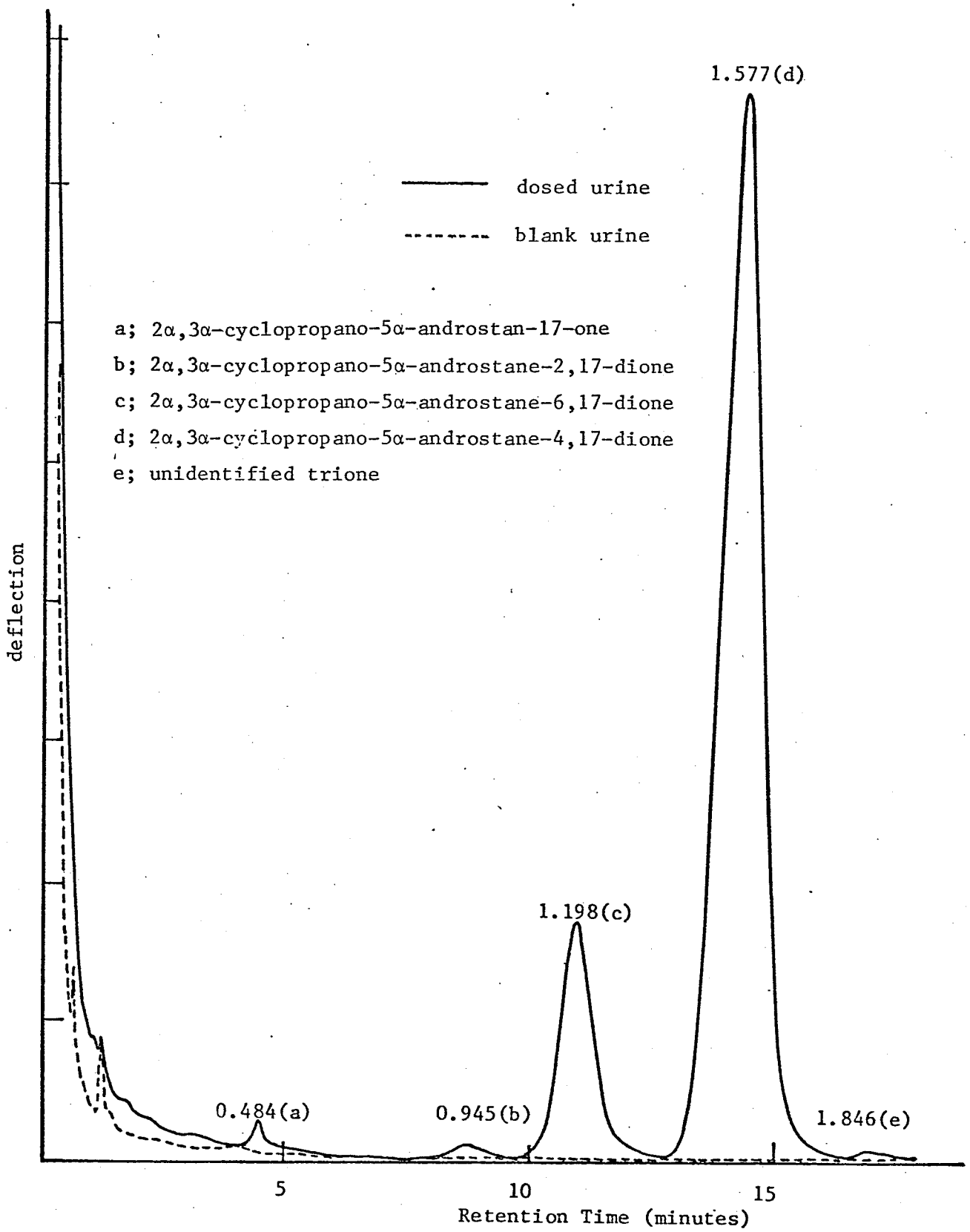


Fig. 21. GLC analysis of the oxidation product of the total urinary extract, after oral administration of II.

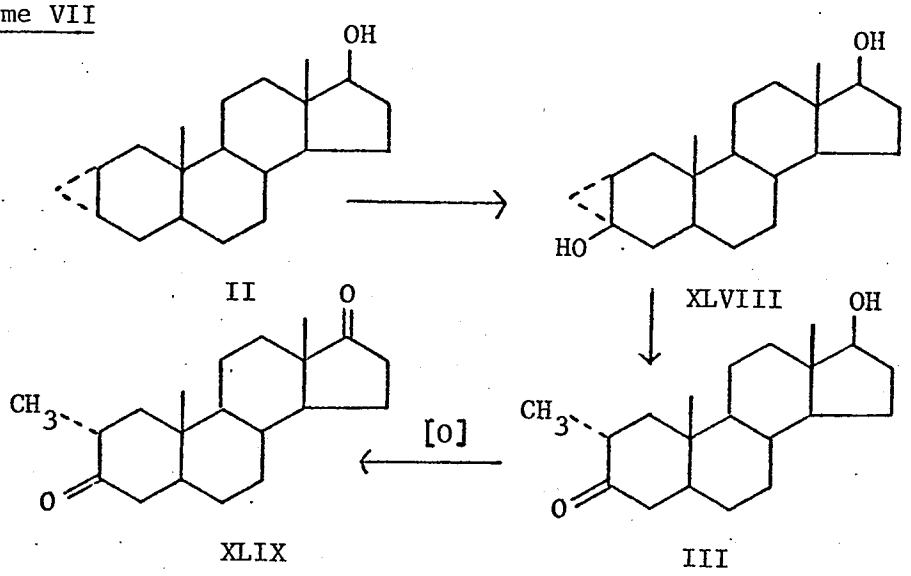
5α-androstan-17-one (0.8%) 3β-methyl-5α-androstane-2,17-dione (XLVII, 1.6%), 2α,3α-cyclopropano-5α-androstane-4,17-dione (XL, 83%), 2α,3α-cyclopropano-5α-androstane-6,17-dione (XLIII, 14%) and an unidentified trione (0.6%). Mass spectra of these peaks collected from the GLC were identical with those of the pure substances. No indication of the presence of 2α-methyl-5α-androstane-3,17-dione was observed. The presence of 17β-hydroxy-2α-methyl-5α-androstan-3-one or its major metabolites was also not detected in the total crude urinary extract (see Experiment C).

The residual 20% of the crude product was not fully identified. A minor peak (RRT=0.350) corresponded to the dosed material (II). The m.s. of this fraction collected from GLC also indicated this substance to be II. A second peak (RRT=0.440) collected from GLC column corresponded to a ketol from its m.s. The remainder appeared to consist of mixture of substances by GLC and TLC corresponding to the more polar column fractions. The m.s. of these column fractions indicated the presence of triols and a diolone which were not obtained in pure states.

In a separate experiment, acid hydrolysis on the total urinary extract was carried out as a preliminary work assuming the proposed metabolic sequence (Scheme VII) was taking place. If so, Jones' oxidation would give 2α-methyl-5α-androstane-3,17-dione(XLIX), an oxidation product of the potential metabolite, III, eliminating the variety of 3- and 17- epimeric alcohol combinations. However, when a portion of the total crude material was oxidized by Jones reagent, many substances, some of which may have been artifacts from the acid treatment, were detected on TLC (8 spots) and GLC (8 peaks), none of

which corresponding to 2α-methyl-5α-androstane-3,17-dione. Chromatography on alumina did not allow separation of these substances.

Scheme VII

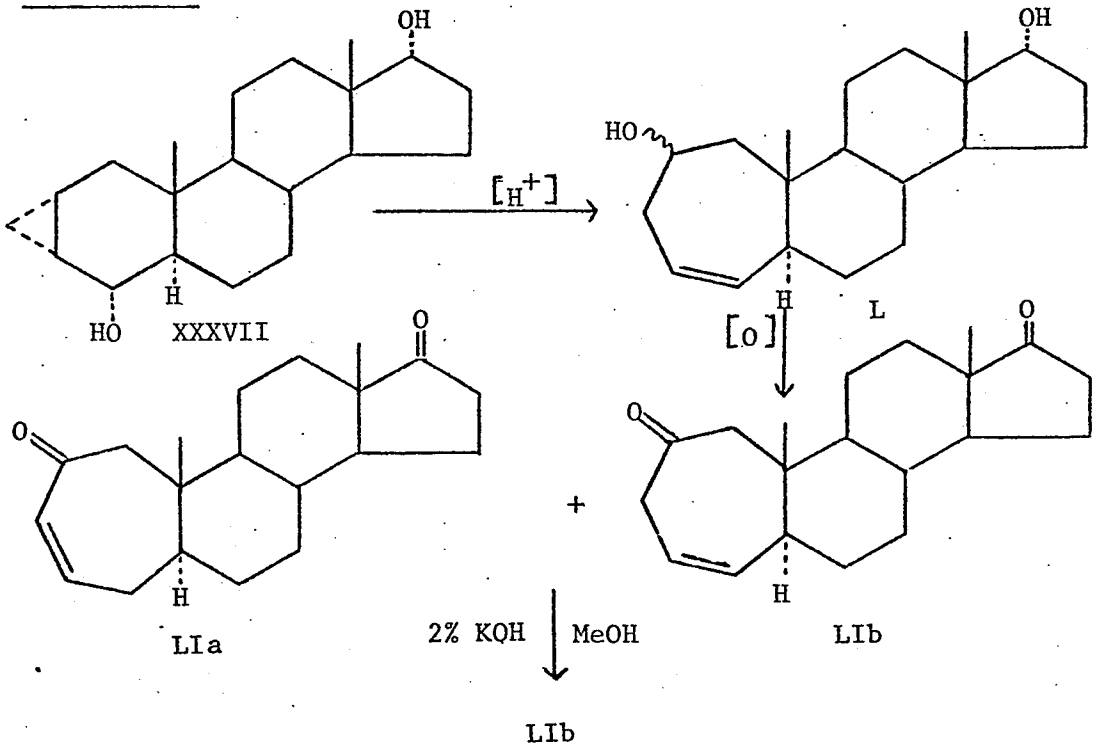


From the non-ketonic portion of the total crude material of acid hydrolysis, a major substance isomeric with the major metabolite (XXXVII) from enzymatic hydrolysis was isolated. This substance was an unsaturated diol no longer containing the cyclopropane ring (i.r. and p.m.r. spectra) and showed two olefinic protons in the p.m.r. spectrum and is in agreement with A-homo-5α-androst-4-ene-2ξ,17α-diol(L), derived from XXXVII by ring expansion as described for analogous steroidal α-cyclopropano alcohols[99].

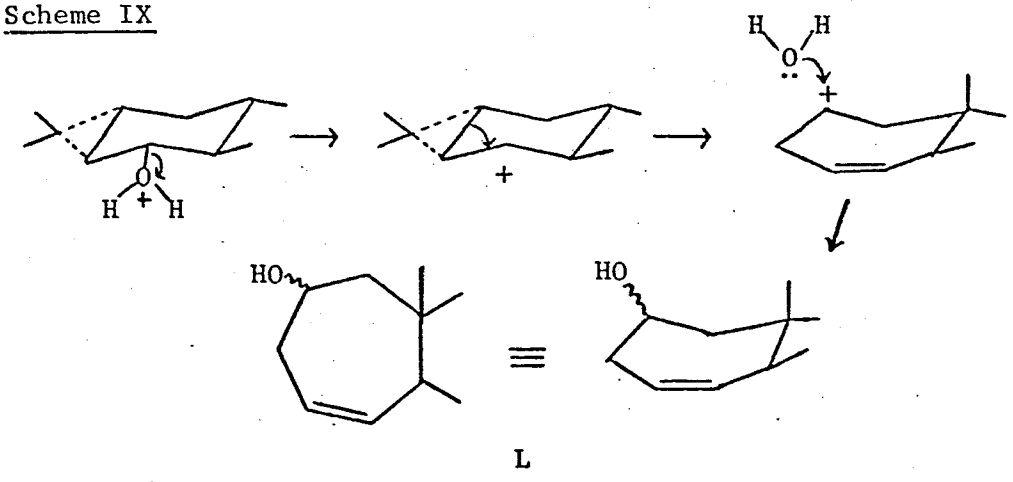
When L was oxidized by Jones reagent, the i.r. spectrum indicated the presence of three carbonyl absorptions at 1742 (C=O, 5-membered ring), 1707(C=O, β,γ-unsaturated in a 7-membered ring) and 1686(C=O, conjugated to a double bond in a 7-membered ring)cm^{-1}. When the oxidized product was treated with 2% KOH/MeOH, the absorption band at 1686 cm^{-1} disappeared in accordance with the known conversion

of a conjugated ketone to the non-conjugated isomer by acid or base treatment[100]. In agreement with the structure of L , oxidation led to a mixture of conjugated and non conjugated ketone and the former was converted to the latter by the base treatment as shown in Scheme VIII. The proposed mechanism of the formation of L from XXXVII is shown in Scheme IX.

Scheme VIII



Scheme IX



Robertson and Dunstan[12] have proposed a framework for the active site of the microsomal hydroxylating enzyme system for the oxidation of some polycyclic hydrocarbons and suggested the extension of this framework to the steroid ring system (see Experiment A). Application of this framework to the hydroxylation products obtained from 17β-hydroxy-2α,3α-cyclopropano-5α-androstan-3-one (II) indicates that the cyclopropane ring projecting below the plane of the steroid nucleus may impede reaction at position 2 and 3 although the presence of some oxidation product derived from hydroxylation at position 2 suggest that this may not be totally exclusive. In terms of the proposed framework for the active site oxidation at carbon 2 also projects carbons 12, 13, 16, 17 and 18 outside the framework.

Here it is possible to suggest that oxidation at carbon 2 may involve a different enzyme system since the β-face geometry and electron density of the cyclopropane ring is analogous to a carbon 2-3 double bond as present in the enolic form of a steroid 3-ketone suggesting an enzyme system capable of attacking an "activated" carbon atom as opposed to oxidation of a saturated C-H σ-bond.

Hydroxylation at the sterically hindered 4-position requires that carbon 7 and the D-ring project outside the proposed framework, however, hydroxylation at carbon 6 requires much less distortion of the framework causing only carbon 3 and 16 to be outside the model.

The generally poor "fit" of the hydroxylation products of II to those predictable from the proposed framework of the active site suggests that extension of this model to the steroid system is of little predictive value.

Whereas the steric requirements of the oxidative enzymes are undoubtedly a major factor in the position of oxidation of foreign molecules, if the biological purpose of oxidation (and conjugation) is to increase water solubility in order to aid excretion from the biological system as is generally accepted, then the position of hydroxylation may also be expected to result in the production of the more water soluble derivative (or conjugate). It follows from the above that oxidation will occur away from any polar function initially present in the molecule since this can be expected to increase the water solubility and decrease the lipid solubility (i.e. increase the water/lipid partition coefficient) more effectively than oxidation at an adjacent site. Oxidation adjacent to an initial polar function may lead to the partial neutralization of the polarity through intramolecular interactions thereby decreasing the relative water solubility. This effect on water solubility can be illustrated with benzoic acid derivatives. Benzoic acid has a solubility of 1 part in 500 parts of water compared with the ortho hydroxy derivative, salicylic acid, which dissolves in 460 parts of water; introduction of the hydroxyl group causing little increase in the water solubility. However, p-hydroxybenzoic acid has a solubility of 1 part in 125 parts of water, a four-fold increase.

Oxidation remote from an initial polar function in a steroid molecule containing a large hydrocarbon portion also may aid excretion by decreasing the possibility of hydrophobic interactions with lipid molecules[101, 102].

The hypothesis that the activity of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (II) may be due to its metabolic transformation

to 17β-hydroxy-2α-methyl-5α-androstan-3-one (III) in light of the equivalent anabolic/androgenic activity exhibited by these compounds is not supported by the results of the experiment. No evidence for the formation of III or its metabolites has been found.

The possibility that the biological activity of II may be due to the metabolites formed has been tested by determining the anabolic/androgenic activity of the 4α- and 6α-hydroxy derivatives isolated as urinary metabolites. No significant activity on ventral prostate, seminal vesicles, levator ani was observed. There was no alteration in adrenal weight.*

Thus the above experiment suggest that the activity of II may be due to the parent molecule rather than an oxygenated metabolite consistent with the view of Wolff[13] that the activity of this molecule is dependent on steric interactions of the cyclopropane ring with a receptor molecule.

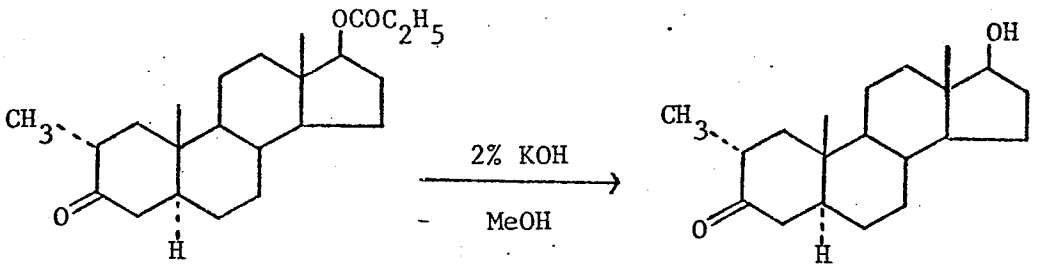
The presence of a large proportion of 17α-hydroxyl epimer in the metabolites compared with the starting 17β-alcohol is not unexpected in experiments conducted in rabbits[103]. Metabolic hydroxylation of an unactivated cyclopropane ring has not, to our knowledge, been previously reported.

* The androgenic-myotropic tests were carried out by Endocrine Laboratories of Madison, Inc., 679 Jonathan Dr., Madison, Wisconsin, 53713.

C. Metabolism of 17β-hydroxy-2α-methyl-5α-androstan-3-one (dromostanolone)

Dromostanolone (III) was prepared by hydrolysis of dromostanolone propionate (LII) as shown in Scheme X.

Scheme X



LII III

The total crude neutral steroid extract, accounting 30% of the dose, from enzymic hydrolysis of the urine from rabbits dosed with dromostanolone (III) was divided into a ketonic and a non-ketonic fraction with Girard T-reagent. The GLC data of the total crude urinary extract, ketonic and non-ketonic fractions are shown in Figs. 22, 23 and 24, respectively.

The ketonic fraction consisted mainly of one substance by TLC and GLC (relative percentage of the peak area, 70%). On column chromatography over alumina a crystalline substance was obtained. The infrared spectrum of this substance showed the presence of hydroxyl absorption and a five-membered ring carbonyl group. The mass spectrum indicated a molecular ion isomeric with dromostanolone. The p.m.r. spectrum showed the presence of a C-18 methyl signal indicative of a 17-carbonyl function and a C-19 methyl signal together with a

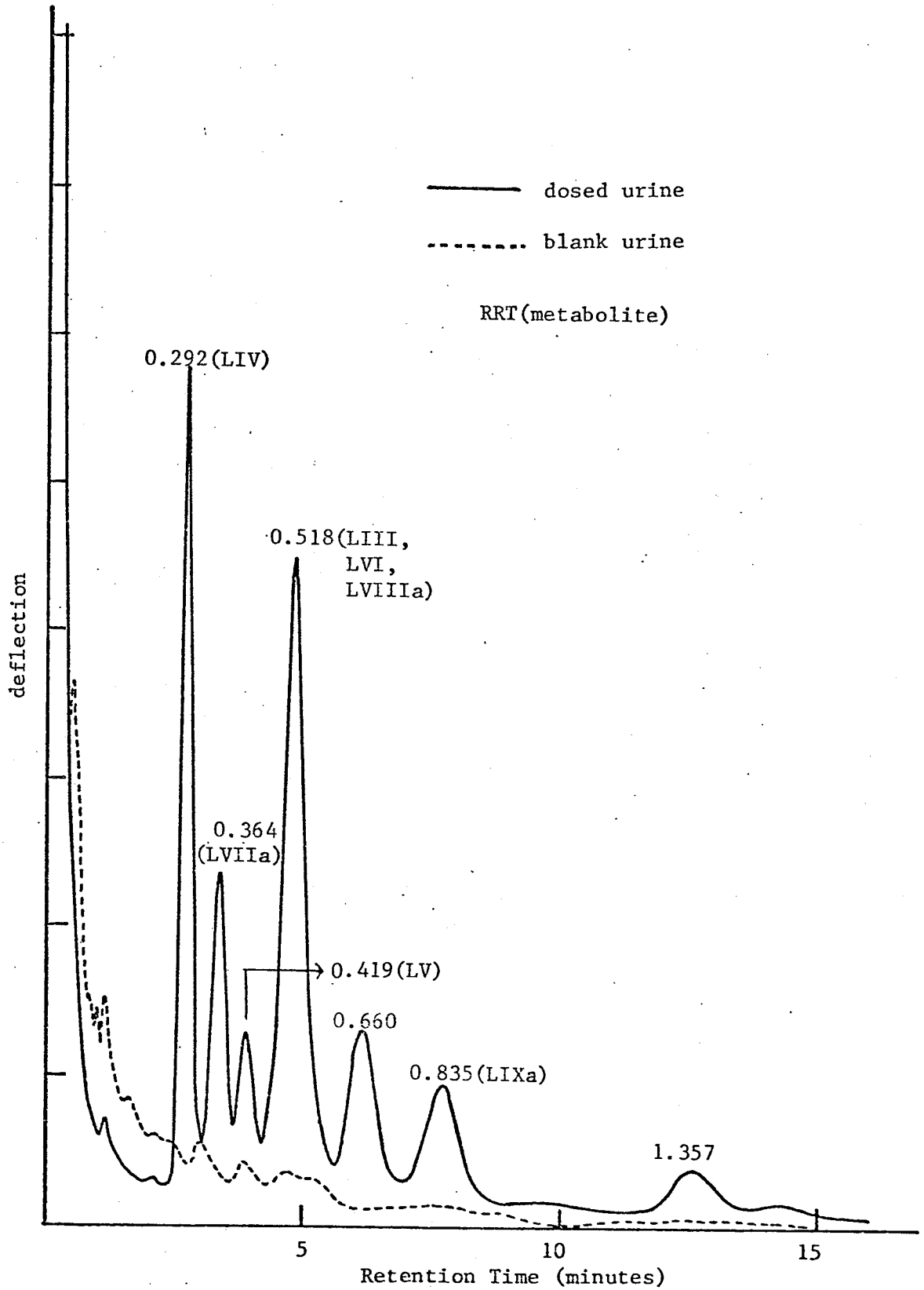


Fig. 22. GLC analysis of the total urinary extract, after oral administration of dromostanolone (III).

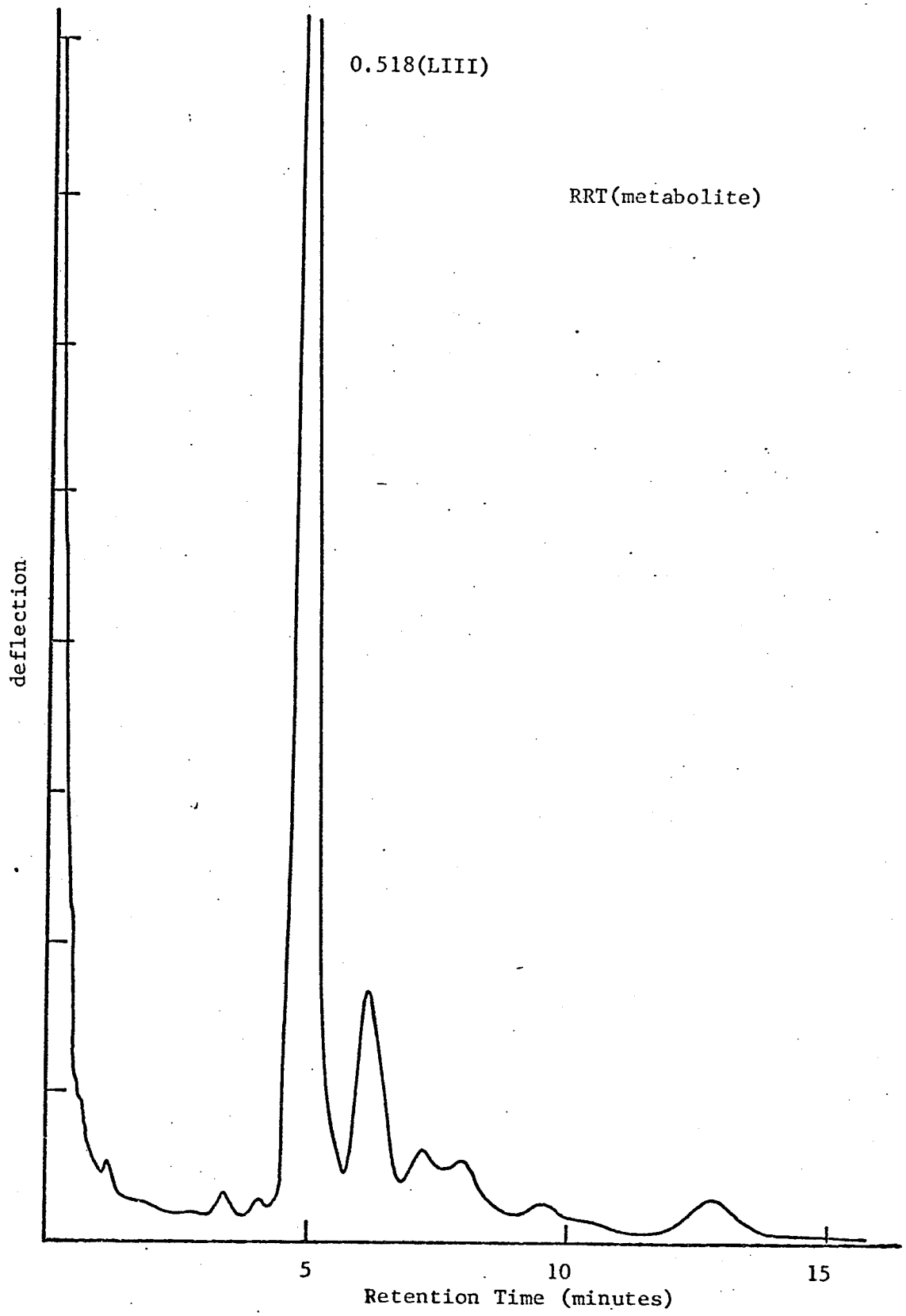


Fig. 23. GLC analysis of the ketonic fraction of the total urinary extract, after oral administration of III.

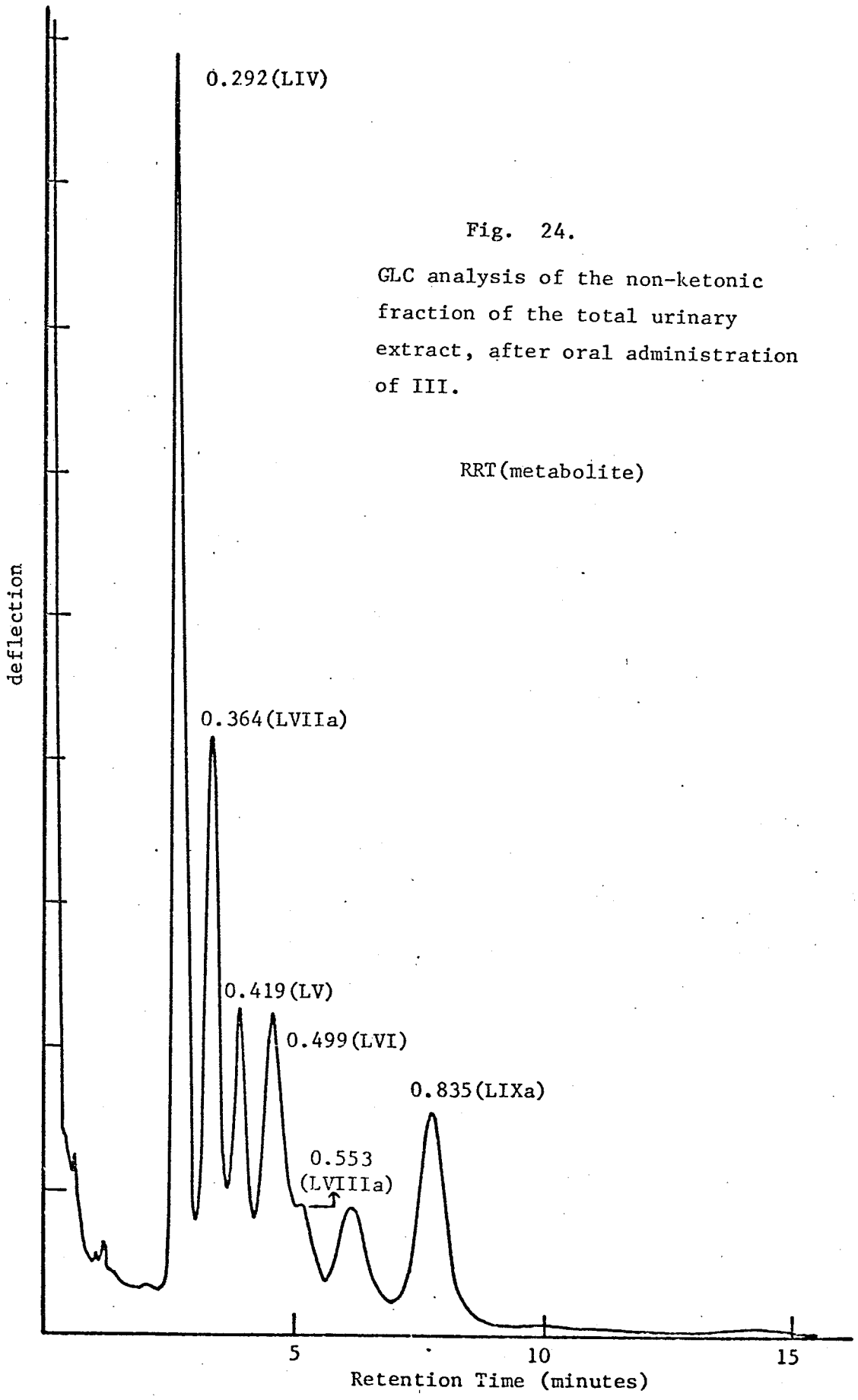


Fig. 24.

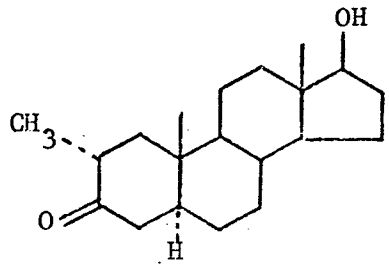
GLC analysis of the non-ketonic fraction of the total urinary extract, after oral administration of III.

RRT(metabolite)

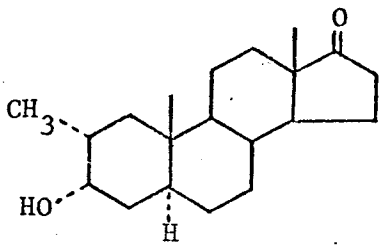
downfield proton (3.77 ppm, $W_{\frac{1}{2}}$=7Hz) in agreement with a 3α-hydroxyl group. A doublet (0.90 ppm) showed the presence of the 2α-methyl function. Jones' oxidation yielded 2α-methyl-5α-androstane-3,17-dione (XLIX) which was identified by comparison with an authentic sample prepared from Jones oxidation of dromostanolone. These results establish this substance as 3α-hydroxy-2α-methyl-5α-androstan-17-one (LIII).

The non-ketonic fraction on column chromatography over alumina yielded fractions from which the following substances have been obtained. Two diol compounds were identified as 2α-methyl-5α-androstane-3α,17α-diol (LIV) and 2α-methyl-5α-androstane-3β,17α-diol (LV) on the following evidence. Both substances on Jones' oxidation yielded 2α-methyl-5α-androstane-3,17-dione (XLIX) thereby establishing the position of the two hydroxyl groups. Both substances showed the presence of a downfield proton (3.69-3.71 ppm, d, J=6Hz, 1H) corresponding to a 17α-hydroxyl group. Each substance showed a second downfield proton having the characteristic chemical shift and splitting pattern of a 3α-hydroxyl (LIV, 3.754 ppm, m, $W_{\frac{1}{2}}$=7Hz) and 3β-hydroxyl (LV, 3.114 ppm, m, $W_{\frac{1}{2}}$=27Hz). Comparison of the TLC, GLC and m.s. of a further fraction suggested the presence of a minor metabolite, 2α-methyl-5α-androstane-3β,17β-diol (LVI).

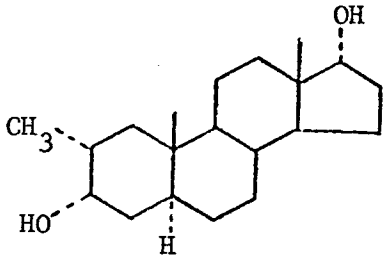
From the more polar fractions 2α-methyl-5α-androstane-3α, 15α,17α-triol (LVIIa) has been identified on the following evidence. Carbon-hydrogen analysis and the mass spectrum (m/e: 304, M^+-H_2O) indicated that this substance was a triol. The p.m.r. spectrum showed the presence of the 2α-methyl function and three downfield



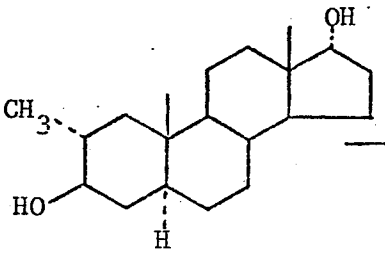
III



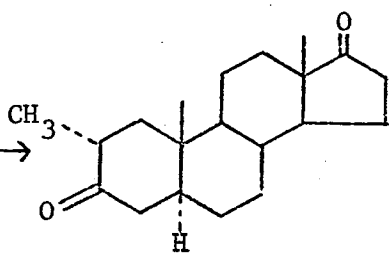
LIII



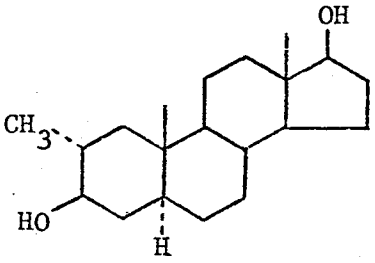
LIV



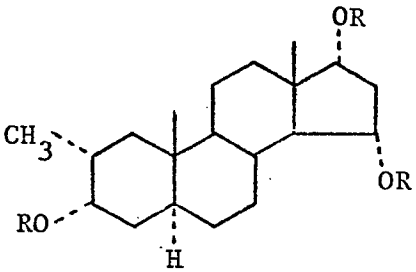
LV



XLIX



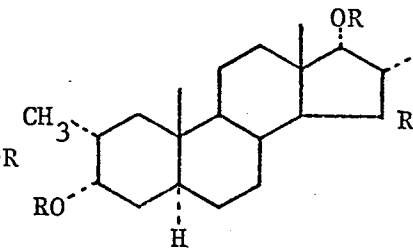
LVI



LVII

a; R = H

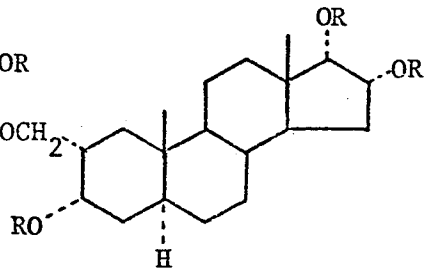
b; R = $COCH_3$



LVIII

a; R = H

b; R = $COCH_3$



LIX

a; R = H

b; R = $COCH_3$

protons. Two of these protons were in agreement by their chemical shifts and splitting patterns with the presence of the 3α- and 17α-hydroxyl functions. The remaining downfield proton was assigned the 15β-configuration on the basis of the chemical shift and splitting pattern. The p.m.r. spectrum of the derived triacetate showed three acetoxymethyl signals and three lower downfield protons two of which are in agreement with the 3α- and 17α-acetoxy derivatives. The third signal which was shifted downfield relative to the hydroxyl group by 0.91 ppm is in agreement with a hydroxyl group in the 5-membered D-ring since shifts on acetylation of <1 ppm have been associated with D-ring hydroxyl functions[104]. The chemical shifts of the C-18 and C-19 although not unique are also consistent with the 15α-hydroxyl group. The C-18 and C-19 methyl shifts clearly eliminate 4β,6β,11α, 11β,15β and 16β-hydroxyl substitution because of the large alteration in their chemical shifts.

Evidence for the presence of a second non-crystalline triol identified as 2α-methyl-5α-androstan-3α,16α,17α-triol (LVIIIa) is based on the following evidence. Chromatographic (TLC, GLC) and mass spectral data (m/e: 322, M^+) indicate this substance is a triol. The p.m.r. spectrum of the alcohol and its derived acetate are in agreement with the presence of the 2α-methyl group together with the 3α- and 17α-hydroxyl functions and one newly introduced hydroxyl function. This hydroxyl group is assigned the 16α-configuration on the basis of the similarity of the band width at half-height[87a] and chemical shift of the 16β-proton. Further the alteration in this proton on acetylation (0.92 ppm) is also consistent with this assignment.

A substance eluted from the most polar fractions and isolated as an impure crystalline solid showed a mass spectrum (m/e: 388, M^+) indicative of a tetrol. Acetylation yielded a non-crystalline product whose increased solubility allowed a p.m.r. spectrum in $CDCl_3$ to be recorded. The p.m.r. spectrum clearly showed the presence of four acetoxyl functions and the absence of a doublet associated with the 2α-methyl group. Three downfield protons were in agreement with the presence of the 3α-, 16α-, and 17α-acetoxyl functions as assigned to the triacetate (LIXb). An ill defined signal at 3.814-3.960 ppm integrating for two hydrogen atoms was assigned to an acetoxymethyl group thereby accounting for the loss of the 2α-methyl signal. This tetrol can be assigned the structure 2α-hydroxymethyl-5α-androstane-3α,16α,17α-triol (LIXa).

The relative percentages of each metabolite was estimated from the GLC peak areas of the total urinary extract, ketonic (39% of the total weight) and non-ketonic fractions (61% of the total weight) as shown in Table 5.

Table 5. The relative percentages of the GLC peak areas of the urinary metabolites of 17β-hydroxy-2α-methyl-5α-androstan-3-one.

Metabolites	%
3α-hydroxy-2α-methyl-5α-androstan-17-one(LIII)	27.4
2α-methyl-5α-androstane-3α,17α-diol(LIV)	20.3
2α-methyl-5α-androstane-3β,17α-diol(LV)	5.6
2α-methyl-5α-androstane-3β,17β-diol(LVI)	6.2
2α-methyl-5α-androstane-3α,15α,17α-triol(LVIIa)	10.8
2α-methyl-5α-androstane-3α,16α,17α-triol(LVIIIa)	2.6
2α-hydroxymethyl-5α-androstane-3α,16α,17α-triol(LIXa)	11.0
others	16.1
Total crude urinary extract	100.0

IV. EXPERIMENTAL

Unless otherwise stated the following instruments and procedures have been used.

Instruments:

Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected.

Optical rotations were measured in chloroform solution at 23° on a Bellingham and Stanley (Model A) Polarimeter.

Infrared (i.r.) spectra were recorded on either a Beckman Model 8 Spectrophotometer or a Perkin-Elmer Model 237 instrument using the KBr disc method.

Proton magnetic resonance (p.m.r.) spectra were obtained in deuterochloroform as solvent and tetramethylsilane as internal standard either on a Varian XL-100-15 spectrometer with accumulations recorded in the CW mode utilizing the Varian 620L Data Machine (Experiment A) or Varian HR 220 instrument (Experiments B and C) by the Canadian 220 MHz NMR Center, Ontario Research Foundation, Sheridan Park, Ontario. Cyclopropane derivatives were recorded initially without the presence of the internal standard. Hydroxylic compounds were recorded in the presence of deuterium oxide. Routine spectra were recorded on a Varian A56/60A instrument.

Mass spectra (m.s.) were determined on a Finnigan Quadrupole Model 1015 instrument at 70 eV using a direct probe method by the Chemistry Department, University of Manitoba. Mass spectra of silylated derivatives were recorded either on the above instrument or on an A.E.I.MS12 instrument at 70eV using a direct probe method by the Chemistry Department, University of Alberta, Edmonton.

Elemental analysis was performed either by Pascher and Pascher, Microanalytical Laboratory, Bonn, West Germany (Experiment A) or Mr. G. Crouch, Microanalytical Laboratory, School of Pharmacy, University of London, England (Experiments B and C).

Chromatography:

Column chromatography was carried out on basic alumina. Thin-layer chromatography (TLC) was run on silica gel coated (0.25 mm thickness) glass plates and developed in the following solvent systems. Petroleum ether (P.E.) refers to the fraction boiling within the range of 60° to 90°.

System Ia; 50% ethyl acetate/petroleum ether

System Ib, 25% ethyl acetate/petroleum ether

System Ic; 10% ethyl acetate/petroleum ether

System II; 5% acetone/$CHCl_3$

System III; 5% MeOH/CCl_4

Plates were visualized by spraying with 4% v/v concentrated sulfuric acid in ethanol followed by heating at approximately 100°. Thick-layer chromatography was run on silica gel coated (1 mm thickness) preparatory plates. After developing in the specified solvent systems, the bands containing desired substances were removed and dissolved in warm MeOH:$CHCl_3$(1:1), filtered through sintered glass with a celite pad and taken to dryness at reduced pressure. The residue was dissolved in ether and filtered through cotton wool and evaporated. The residue obtained was applied on TLC to determine their complete separation. Silver nitrate treated plates were prepared by spraying the above plates with 0.1 N silver nitrate and drying at 100° for 30 minutes before use.

Gas-liquid chromatography (GLC) was recorded on a Varian Aerograph Ser. 1800 chromatograph equipped with a hydrogen flame ionization detector and a pyrex glass column packed with 2% OV-17 on Chromosorb GHP, 80/100 mesh, internal diameter 2 mm, length 180 cm. For the study of metabolism of 5α-androstan-3-one, the detector temperature was 300°, column temperature 240° and carrier gas (N_2) flow rate was 40 ml/min. For others, the detector temperature was 275°, column temperature 240° and the carrier gas (N_2) flow rate was 30 ml/min. The latter condition was also employed for the compounds, where mass spectrometry was performed on the effluents of the column using a capillary collector attached to the GLC stream splitter. All steroids were treated with trimethylsilyl chloride in pyridine before injection so that 1 µl contained 25 µg of steroid. Retention times are relative to 5α-cholestane. The GLC response (peak area) was calibrated against each pure metabolite, using known amount of 5α-cholestane as an internal standard to avoid any error due to injection volume[105]. In all cases, straight lines passing through the origin were obtained indicating that GLC response was proportional to the amount of metabolites present.

Materials

Rabbits were purchased from Canadian Breeding Laboratories, 188 La Salle, St. Constant, P.Q.

β-Glucuronidase (Type B-I, bovine liver) with an activity of 800,000 Fishman units/g was supplied by the Sigma Chemical Company, St. Louis, Missouri.

Sulfatase (Type H-1), partially purified powder from Helix Pomata, was also supplied from the above company. 1 g of sulfatase hydrolyzes approximately 15,000-40,000 µ moles of p-nitrocatechol

sulfate per hr. at pH 5.0 at 37°C. Sulfatase also contains approximately 300,000 Fishman units of β-glucuronidase activity per gram.

Trimethylsilyl derivatives of steroid compounds for GLC were prepared by stirring with TRI-SIL, supplied by Pierce Chemical Company, Rockford, Illinois.

Alumina (Activity II) was supplied by British Drug Houses (B.D.H.). Ethyl acetate washed alumina was prepared by stirring the above alumina with ethyl acetate, letting the mixture stand for 1-2 days, filtering, and drying at 80°(106).

Zinc-copper couple was prepared according to the method of Templeton & Wie(116).

General procedures:

Isolation of Steroids;

When the reaction was completed it was usually stopped by pouring the reaction mixture into an excess of ice-water. The resulting slurry was then extracted with an appropriate organic solvent (ether, CH_2Cl_2, or $CHCl_3$). The organic layer was washed with water, with saturated aqueous sodium bicarbonate (if acidic material may be present), with dilute HCl (if basic material may be present), again with water, and finally with saturated aqueous sodium chloride solution. The organic solution was dried over anhydrous sodium sulfate, filtered and taken to dryness at reduced pressure. The residue depending upon its purity was recrystallized or chromatographed.

Enzyme (β-glucuronidase) hydrolysis;

Urine was adjusted to pH 5.0 with glacial acetic acid and incubated with bovine liver β-glucuronidase (800 Fishman units/ml urine) at 37° for 72 hr. and extracted with ether. The combined ether

layers were washed successively with 0.5N-HCl, 1N-NaOH, and water and worked up as described above. Urine incubated for 6 days with the enzyme showed an identical GLC pattern with that incubated for 3 days.

Separation of steroid into ketonic and non-ketonic portions[86];

A solution of steroid (3.8 mmoles) and Girard T-reagent (4.2-4.5 mmoles) in 1M-HOAc/95%EtOH(12.5 ml) was heated to reflux for 1 hr. The reaction was cooled, poured into excess cold saturated aqueous sodium bicarbonate and extracted with ether. Ether layers were washed with bicarbonate solution, followed by the general work-up procedure to give a crude non-ketonic fraction. The combined sodium bicarbonate extracts and washings were acidified with concentrated hydrochloric acid, heated on the steam-bath for 30 min., cooled, extracted with ether and worked up as before to give a crude ketonic fraction. Equivalent control experiment was carried out on a blank urine and the result compared with dosed urine by TLC and GLC.

Chromic acid/acetone oxidation (Jones oxidation)[85];

Steroid (0.3 mmoles) was dissolved in acetone (2 ml) and excess Jones reagent (8N-chromic acid)(0.2 ml) was added with stirring at 15-20° by titration until a brown color was obtained. The excess reagent was reacted with MeOH by dropwise addition until the color changes to green. Water was added and the reaction mixture was extracted with ether, washed with saturated aqueous bicarbonate and worked up in the usual way. Equivalent control experiment was carried out on a blank urine and the result compared with the dosed urine (TLC, GLC).

Acetylation;

To a 10% solution of the steroid in dry pyridine was added one-half the volume of acetic anhydride. After standing overnight at room-temperature, the reaction was poured into an ice-water mixture and allowed to stand for 30 min. followed by acidification with dilute hydrochloric acid and ether extraction.

Administration of steroids;

Mature male albino rabbits (2.5-3.5 kg), maintained on a Purina rabbit chow diet and water ad libitum, were housed singly in cages designed for efficient separation of urine and faeces. A controlled illumination environment of 12 hr. light and 12 hr. darkness was maintained. A finely divided slurry of steroid in propylene glycol was administered orally by stomach tube and the tube was rinsed with 2 ml of propylene glycol. Urine was collected until 72 hr. after the last dose under a layer of toluene. Daily collected urine was pooled and stored at -5°. The crude residue obtained from the freshly collected urine showed an identical GLC pattern to the residue obtained from urine which was left at room-temperature for seven days under a layer of toluene.

Control experiments were performed, and urine collected over an appropiate period, under identical conditions to those obtaining in the dosed experiments. The same rabbits were used in the control experiments prior to dosing. Two control experiments were carried out. In the first case urine was collected from the untreated animals. In a second experiment urine was collected from rabbits dosed with pure propylene glycol in the same amounts and intervals used in the dosed

experiments. In repeated experiments with different animals no significant differences in weights of total crude neutral residue, GLC and TLC were observed between the untreated control and the propylene glycol control. Thus the latter will be refered as blank urine.

A. Metabolism of 5α-androstan-3-one

Preparation of 5α-androstan-3-one;

A mixture of 3β-hydroxy-5α-androstan-17-one (XXIV) (10g), potassium hydroxide (8g), and hydrazine hydrate (5 ml) in ethylene glycol (75 ml) was heated to reflux for 1 hr. The internal temperature was raised to 200° by distillation and held there for 2½ hr. under reflux. On addition of water (500 ml) a precipitate formed which was extracted into ether and worked-up. The recrystallization of the residue from acetone gave 5α-androstan-3β-ol (XXV) (7.8 g), m.p. 149-151° (lit.[107] 147.5-148°).

5α-Androstan-3β-ol (XXV) (10 g) was dissolved in acetone (200 ml) and oxidized with an excess of Jones reagent (20 ml). The crude product was recrystallized twice from hexane to afford 5α-androstan-3-one (I) (7.9g), m.p. 99.5-100°; $[\alpha_D]$ +21, (c, 1, dioxane), lit.[108] m.p. 104.5-105.5°; $[\alpha_D^{24}]$ +18, (c, 1, dioxane). The steroid was chromatographically pure (TLC, GLC).

Administration of 5α-androstan-3-one

i) Two rabbits were each dosed (1g) three times at two-day intervals with 5α-androstan-3-one. Urine (4000 ml) was collected for a total of seven days. Blank urine (3400 ml) was collected prior to dosing. Similar results were obtained in three experiments using different animals.

ii) In a separate experiment, one-tenth of the previous dose (100 mg) was administered to the rabbits as above and urine was collected over seven days.

iii) Rabbits were treated with neomycin sulfate, U.S.P. twice a day (1 g in 5 ml of water) for two days before administering a dose of 100 mg of 5α-androstan-3-one as above.

Isolation of neutral steroids

Acid hydrolysis: A portion (2 l) of the total urine collected from the above 1 g dose (i) was acidified to 1N with conc. HCl and heated to reflux. The cooled solution was extracted with ether (250 ml, x4) and the combined ether layers were washed with aqueous 2N-NaOH followed by general work-up procedure to give crude gummy residue (792 mg). Equivalent control experiments gave 116 mg and 126 mg of crude residue when dosed with and without propylene glycol, respectively. The results from 1g dose (i) and 100 mg dose (ii) showed only quantitative differences in the relative intensities of the five major metabolites on GLC. Urine from animals pretreated with neomycin sulfate showed an identical GLC trace for the five major peaks as obtained in the equivalent experiment without neomycin treatment.

Enzyme Hydrolysis: A portion (25 ml) of urine from 100 mg dose (ii) was hydrolyzed by β-glucuronidase as described before. Crude residue (22 mg) was obtained which showed an identical GLC pattern as the one obtained from acid hydrolysis.

Isolation of free steroids: A portion (36 ml) of unhydrolyzed urine from 100 mg dose was extracted with ether to isolate any non-conjugated polar metabolites (free steroids). Crude material (26 mg)

was obtained which showed negligible amount of steroidal metabolites on GLC.

Separation of ketonic and non-ketonic materials

A solution of the crude urine extract (1.12 g) was treated with Girard T-reagent to give a crude non-ketonic fraction (700 mg) and a ketonic fraction (410 mg).

Non-ketonic fraction;

5α-Androstane-3β,17α-diol(XXVI)

The non-ketonic fraction from two of the above reactions (1.4 g) showed the presence of three peaks on GLC (RRT, 0.34, 0.43, 0.47). The TLC showed two major spots with R_f of 0.28 and 0.20. The total crude non-ketonic fractions were dissolved in benzene and was placed on the column consisting 50 g of alumina. The least polar substance (R_f, 0.28, System Ia; RRT, 0.43) was eluted from 10-25% ether/benzene (200 mg) and combined fractions were recrystalized several times from either methanol or ethyl acetate to give 5α-androstane-3β,17α-diol(XXVI) (24 mg), m.p. 210-211° (lit,[109] m.p. 214°); i.r. ν max 3333 (OH), 1044; 1076 (CO) cm^{-1}; p.m.r. δ: 0.655 (s, C-18), 0.815(s, C-19), 3.60(m, 3α-H), 3.72(d, J=6Hz, 17β-H) ppm; m.s., m/e: 292(M^+). Comparison of the i.r. and m.s. spectra of this material with an authentic sample showed them to be identical. A mixed melting point was not depressed.

5α-Androstane-3β,16α-diol(XXVIII)

The more polar substance was eluted from 75% ether/benzene and ether (340 mg) which was recrystallized from acetone to give 5α-androstane-3β,16α-diol (XXVIII)(35 mg), m.p. 186-188° (lit[110] m.p.

192-193°); R_f, 0.20(System Ia); RRT, 0.47; i.r. ν max 3390, 3425 (OH), 1040 (CO) cm^{-1}; p.m.r. δ: 0.72(s, C-18), 0.82(s, C-19), 3.60(m, 3α-H), 4.47(m, 16β-H) ppm; m.s., m/e: 292(M^+). Mixed melting point with an authentic sample was undepressed.

5α-Androstane-3α,16α-diol(XXVII)

The most polar substance was eluted from 2.5% MeOH/ether (338 mg) which was unseparated on TLC (R_f, 0.20, System Ia) from 5α-androstane-3β, 16α-diol. Recrystallization from MeOH gave 5α-androstan-3α-16α-diol (XXVII)(67 mg), m.p. 212°; $[\alpha_D]$ - 12.5° (C.1, dioxane); RRT, 0.34; i.r. ν max 3279, 3400 (OH), 1001, 1042 (CO) cm^{-1}; p.m.r. δ: 0.725(s, C-18), 0.805(s, C-19), 4.07(m, 3β-H), 4.49(m, 16β-H) ppm; m.s., m/e: 292(M^+).

Anal. Found: C, 78.17; H, 10.91. $C_{19}H_{32}O_2$ requires C, 78.03; H, 11.03.

Oxidation of 5α-androstane-3α,16α-diol with Jones reagent gave 5α-androstane-3,16-dione (XXXI), m.p. 159-161° (lit.[111] m.p. 157-159°); i.r. ν max 1700 (6-membered ring C=O), 1736 (5-membered ring C=O) cm^{-1}. Mixed melting point with an authentic sample showed no depression.

Ketonic fraction;

3β-Hydroxy-5α-androstan-16-one (XXIX)

The crude ketonic fraction showed on GLC two major peaks (RRT, 0.52, 0.68). Chromatography over alumina gave on elution with benzene and ether/benzene two major fractions. The more polar material (R_f, 0.41 System Ia) on recrystallization from methanol gave 3β-hydroxy-5α-androstan-16-one (XXIX) (16 mg), m.p. 186-187° (lit.[112]

m.p. 186-187°); RRT, 0.68; i.r. ν max 3509 (OH), 1733 (5-membered ring C=O), 1050, 1080 (CO) cm^{-1}; p.m.r. δ: 0.865(s, C-19), 0.89(s, C-18), 3.65(m, $W_{\frac{1}{2}}$=22Hz, 3α-H) ppm. Mixed melting point with an authentic sample was not depressed. Comparison of i.r. and m.s. spectra showed them to be identical.

3α-Hydroxy-5α-androstan-16-one (XXX)

The less polar material (R_f, 0.47 System Ia), was eluted from 5-10% ether/benzene and yielded on crystallization from methanol 3α-hydroxy-5α-androstan-16-one (XXX), m.p. 151-152.5° (13 mg), lit.[113] m.p. 153.5-154.5°); RRT, 0.52; i.r. ν max 3570 (OH), 1733 (5-membered ring C=O), 1000, 1032 (CO)cm^{-1}; p.m.r. δ: 0.83(s, C-19), 0.89(s, C-18), 4.06(m, $W_{\frac{1}{2}}$=7Hz, 3β-H) ppm. Mixed melting point with an authentic sample was not depressed and spectral comparisons (i.r. and m.s.) were identical.

B. Metabolism of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane.

Preparation of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane-2α-bromo-17β-acetoxy-5α-androstan-3-one (XXXIII)[114]

A molar equivalent of dihydrotestosterone (XXXII) (27.8 g) was dissolved in glacial acetic acid (1000 ml) and bromine solution (5 ml ≡ 15.3 g in 100 ml of HOAc) was added dropwise to the vigorously stirred solution. After 24 hr. the reaction was poured into ice-water (6 l) and the precipitate was filtered and washed thoroughly with water. The wet solid was dissolved in $CHCl_3$ and the $CHCl_3$ layer filtered through a fluted filter paper to remove moisture and taken to dryness. The residue was dissolved in hot ether (∿1 l) and abs. EtOH (300 ml) added. Evaporation of the ether gave a slurry of small needles, which after cooling, was filtered, and the solid washed with cold 95% EtOH, 22.7 g; m.p. 173-174° (lit.[115] m.p. 177°); i.r. (Nujol) 1723 (C=O) cm^{-1}.

2α-Bromo-5α-androstane-3ξ,17β-diol-17-acetate (XXXIV)

To a slurry of 2α-bromo-17β-acetoxy-5α-androstan-3-one (XXXIII) (22.3 g, 0.054 moles) in abs. EtOH (4-500 ml) was added dropwise a solution of sodium borohydride (2.056 g, 0.054 moles) in abs. EtOH (100 ml) and the mixture stirred at room-temperature for 1 hr. The reaction was poured carefully into ice-water containing a small excess of mineral acid. The precipitate was filtered, washed thoroughly with water and air dried. i.r. (CCl_4), 3575 (OH), 1727 (acetate C=O) cm^{-1}; p.m.r. δ: 0.788(s, C-19), 0.852(s, C-18), 2.048(s, 17β-OAc), 4.591(t, J=8Hz, 17α-H) ppm.

17β-Acetoxy-5α-androst-2-ene (XXXV)

Freshly prepared 2% zinc-copper couple (142.7 g)[116] was

added to a slurry of 2α-bromo-5α-androstan-3ξ,17β-diol-17-acetate (XXXIV) (22 g) in abs. EtOH (250 ml) and the mixture mechanically stirred at room temperature for 1 hr. TLC indicated that the reaction was complete. The reaction was filtered with suction and zinc-copper couple washed with ether. The filtrate was diluted with excess water and the ether layer separated. The ether was washed with dilute mineral acid, aqueous $NaHCO_3$ and worked up in the usual way. The residue was dissolved in benzene: petroleum ether (1:2) and poured into a column of Al_2O_3 (Shawinigan, 500 g) and eluted with benzene: petroleum ether (1:2) to yield IV (10.5 g), m.p. 97-98°, lit., m.p. 96°(117), 94-95°(118), 101-102°(119); R_f, 0.48 (System Ic); i.r. (CCl_4), 3020 (vinylic C-H str.), 1727 (acetate C=O)cm^{-1}; p.m.r., δ: 0.750(s, C-18 and C-19), 1.946(s, 17β-OAc), 4.49(t, J=8 Hz, 17α-H), 5.53(m, 2H, olefinic protons)ppm.

17β-Acetoxy-2α,3α-cyclopropano-5α-androstane (XXXVI)

Zinc dust (29 g, 0.44 mole) and cuprous chloride (43.6 g, 0.44 mole) in dry ether (75 ml) were mechanically stirred under reflux in a nitrogen atmosphere for 30 min. Dry 17β-acetoxy-5α-androst-2-ene (7 g, 0.022 mole) was added in dry ether (50-75 ml) followed by dropwise addition of methylene iodide (58.8 g ≡ 17.8 ml, 0.22 mole)(120). Reflux was continued and TLC ($AgNO_3$ treated SiO_2 plates in 10% EtOAc/ petroleum ether) at hourly intervals indicated the reaction was complete in 5 - 7 hrs. The reaction was diluted with ether and filtered. The filtrate was added carefully portionwise with stirring to an excess of cold aqueous $NaHCO_3$ (750 ml). The ether layer was separated, washed with dilute aqueous $NaHSO_3$ and treated in the usual

way to give a crude residue which on crystallization from ether/methanol gave the cyclopropane derivative (XXXVI), 4.6 g; m.p. 103-104°, (lit. m.p. 105-6°)[13], i.r. (CCl_4), 3077 (cyclopropane ring), 1734 (acetate C=O) cm^{-1}; p.m.r. δ: 0.727(s, C-18), 0.783 (s, C-19), 1.94(s, 17β-OAc), 4.51(t, J=8Hz, 17α-H) ppm.

17β-Hydroxy-2α,3α-cyclopropano-5α-androstane (II)

17β-Acetoxy-2α,3α-cyclopropano-5α-androstane (4.67 g, 0.0155 moles) was refluxed with 2% (0.36 N) methanolic KOH (92 ml, 0.031 moles) for 1 hour. The reaction was concentrated at reduced pressure to 50 ml, cooled and water added slowly, dropwise with stirring until crystallization was complete. The mixture was cooled in an ice bath, filtered and thoroughly washed with cold MeOH : H_2O (1:1) to give the final product (II), 4.1 g; m.p. 129-130°, lit. m.p. 127-128°[121] and 130-132°[13]; R_f, 0.6 ($AgNO_3/SiO_2$ plate, System Ia); RRT, 0.350; i.r. (CCl_4), 3620 (OH), 3052 (w, cyclopropane ring); p.m.r. δ: 0.167(m, 1H, Cyclopropyl H), 0.670(s, C-18), 0.791(s, C-19), 3.53(t, J=8Hz, 1H, 17α-H) ppm; m.s., m/e: 288(M^+).

2α,3α-Cyclopropano-5α-androstan-17-one

Jones oxidation of II yielded 2α,3α-cyclopropano-5α-androstan-17-one, m.p. 95-96°, (lit.[13] m.p. 97-100°); R_f, 0.784(System Ia); RRT 0.494; i.r. (CCl_4) ν max 3067 (cyclopropyl C-H), 1739(C=O, 5-membered ring) cm^{-1}; m.s., m/e: 286(M^+).

The following comparison compounds were prepared as described in detail in Experimental C.

17β-Hydroxy-2α-methyl-5α-androstan-3-one (III)

17β-Hydroxy-2α-methyl-5α-androstan-3-one (dromostanolone) was prepared by hydrolysis of dromostanolone propionate.

2α-Methyl-5α-androstane-3β,17β-diol (LVI)

LVI was prepared by sodium borohydride ($NaBH_4$) reduction of dromostanolone.

2α-Methyl-5α-androstane-3,17-dione (XLIX)

XLIX was prepared by Jones oxidation of dromostanolone.

Administration of 17β-hydroxy-2α,3α-cyclopropano-5α-androstane

Three rabbits were each dosed (0.7g) three times at two-day intervals with propylene glycol (10 ml) by oral administration. Urine was collected for a total of seven days.

Treatment of Urine

The pooled urine (2900 ml) was treated as follows:

i) A portion (100 ml) was acidified to 1N with conc. HCl and heated to reflux for ½ hr. The cooled solution was extracted with ether followed by usual work-up procedure. A blank urine was treated the same way. Large scale acid hydrolysis was carried out in a separate experiment as a preliminary experiment. This will be discussed in detail.

ii) A portion (100 ml) was hydrolyzed with β-glucuronidase and extracted with ether and worked up in the usual way. A control

experiment was also carried out.

iii) An untreated portion (100 ml) was extracted with ether. Half of the ether extract was washed with 0.5N-HCl, followed by 1N-NaOH and the other half was washed only with 1N-NaOH. The examination of GLC of each work-up residue did not show the presence of non-conjugated metabolites. The results of (i), (ii) and (iii) are summarized in Table 6.

Table 6. The comparison of weights of crude urinary extract from rabbits dosed with II (mg per 100 ml urine)

	Acid hydrolysis	Enzyme hydrolysis	Ether extract (free steroids)	
Blank urine	7.9	8.8	[a] 6.4	[b] 5.0
Steroid dosed urine	88.2	68.8	9.4	5.4

a. Washed with 0.5N-HCl, followed by 1N-NaOH

b. Washed with 1N-NaOH only.

iv) A portion (50 ml) of urine was incubated with sulfatase (0.1345 g) for 5 days, extracted with ether and worked up as before (ii) to yield 26.3 mg of crude residue. The GLC pattern of the crude residue showed identical peaks to those obtained from β-glucuronidase hydrolysate.

v) A portion of urine (250 ml) was hydrolyzed with β-glucuronidase and extracted with ether as (ii). The ether layer was washed with 2N-NaOH and the aqueous layer neutralized and extracted with ether to give 86 mg of crude material. A blank urine (380 ml) was treated in the same way and 57.7 mg of crude residue was obtained from the sodium hydroxide layer.

vi) A portion (35 mg) of total crude urinary extract was oxidized with Jones reagent[85] to give 24 mg of crude material. The equivalent blank experiment gave 36 mg of oxidized product from 88 mg of crude urinary extract obtained from blank urine.

vii) A portion (118 mg) of total crude urinary extract was treated with 2% KOH/MeOH (30 ml) and refluxed for 2 hrs. The reaction mixture was concentrated to half volume and extracted with ether. The ether layer was washed with 0.5N-HCl and worked up in the usual way. The residue (101 mg) was oxidized with Jones reagent to give 86.8 mg of crude material.

A portion (18.5 mg) of the total crude material obtained from the blank urine was treated as above to give 12 mg of crude product which upon Jones oxidation gave 9 mg of product.

viii) A portion (36 mg) of total crude urinary metabolite was treated with Girard T-reagent[86] as described above. Work-up of the reaction mixture gave non-ketonic (21.3 mg) and ketonic (7 mg) materials. An equivalent control experiment (114 mg) gave non-ketonic (77.8 mg) and ketonic materials (36.5 mg).

Isolation of Neutral Steroids (enzymatic hydrolysis):

Since the result of TLC and GLC of (i) and (ii) were quite different, the remaining urine (2250 ml) was hydrolyzed with β-glucuronidase to give a crude neutral steroidal material (1.683 g). The examination of GLC of the silylated total crude extract showed the presence of seven peaks which are not present in the control experiment. Figs. 25, 26 and 27 represent GLC traces of the total crude urinary extract, its ketonic and non-ketonic fractions, respectively, after Girard T separation (see above).

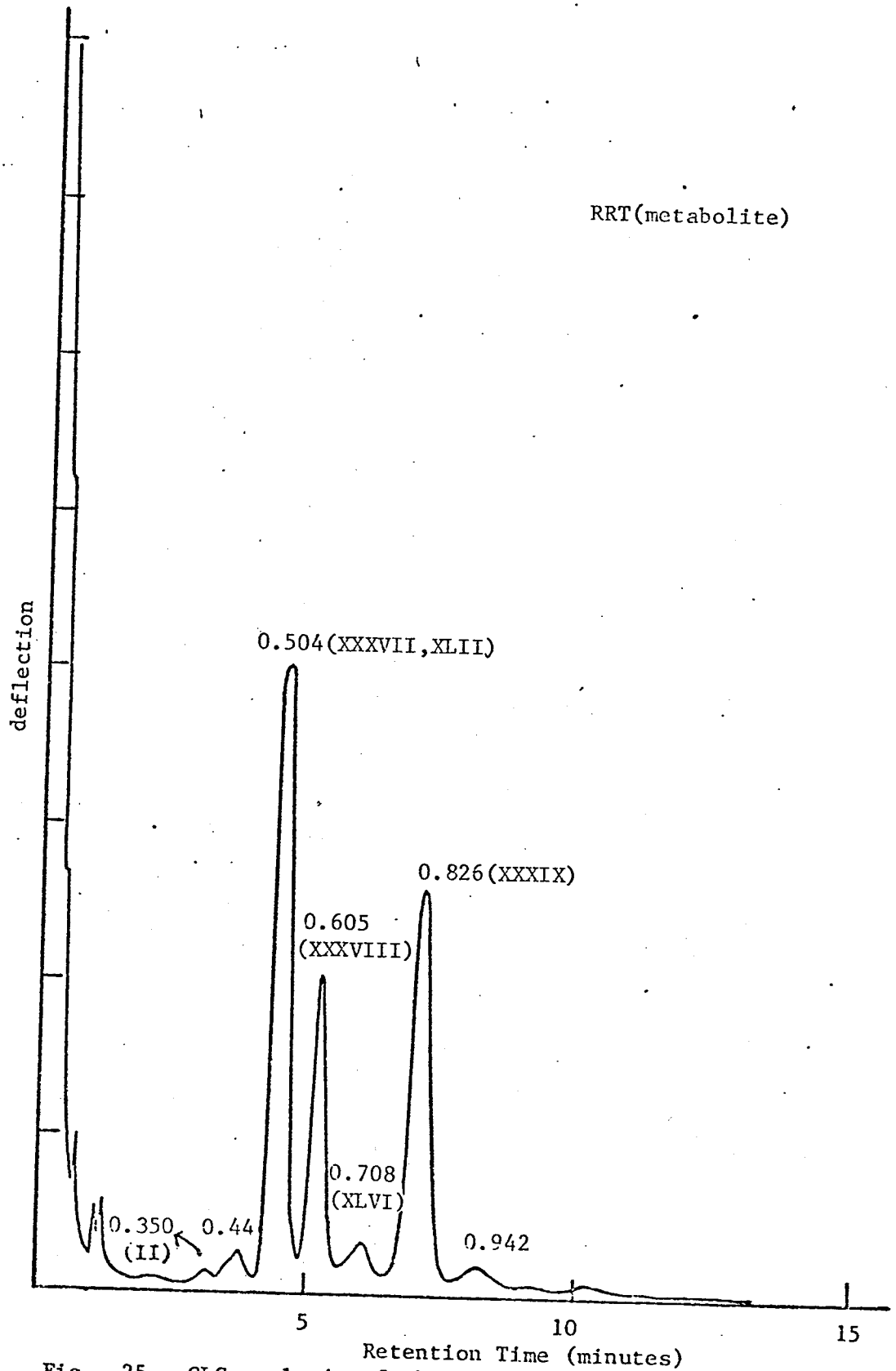


Fig. 25. GLC analysis of the total urinary extract, after oral administration of II...(b).

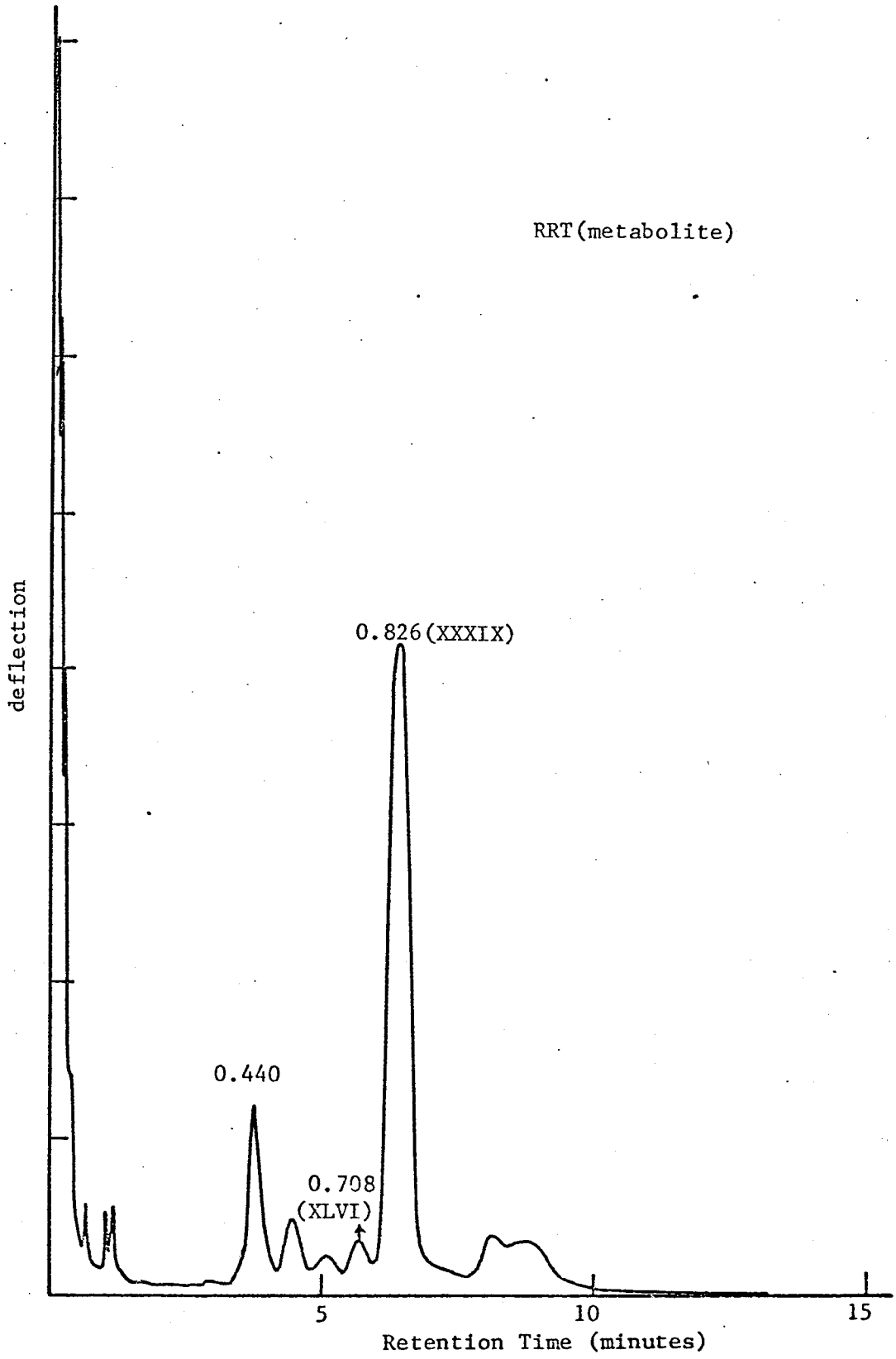


Fig. 26. GLC analysis of the ketonic fraction of the total urinary extract, after oral administration of II.

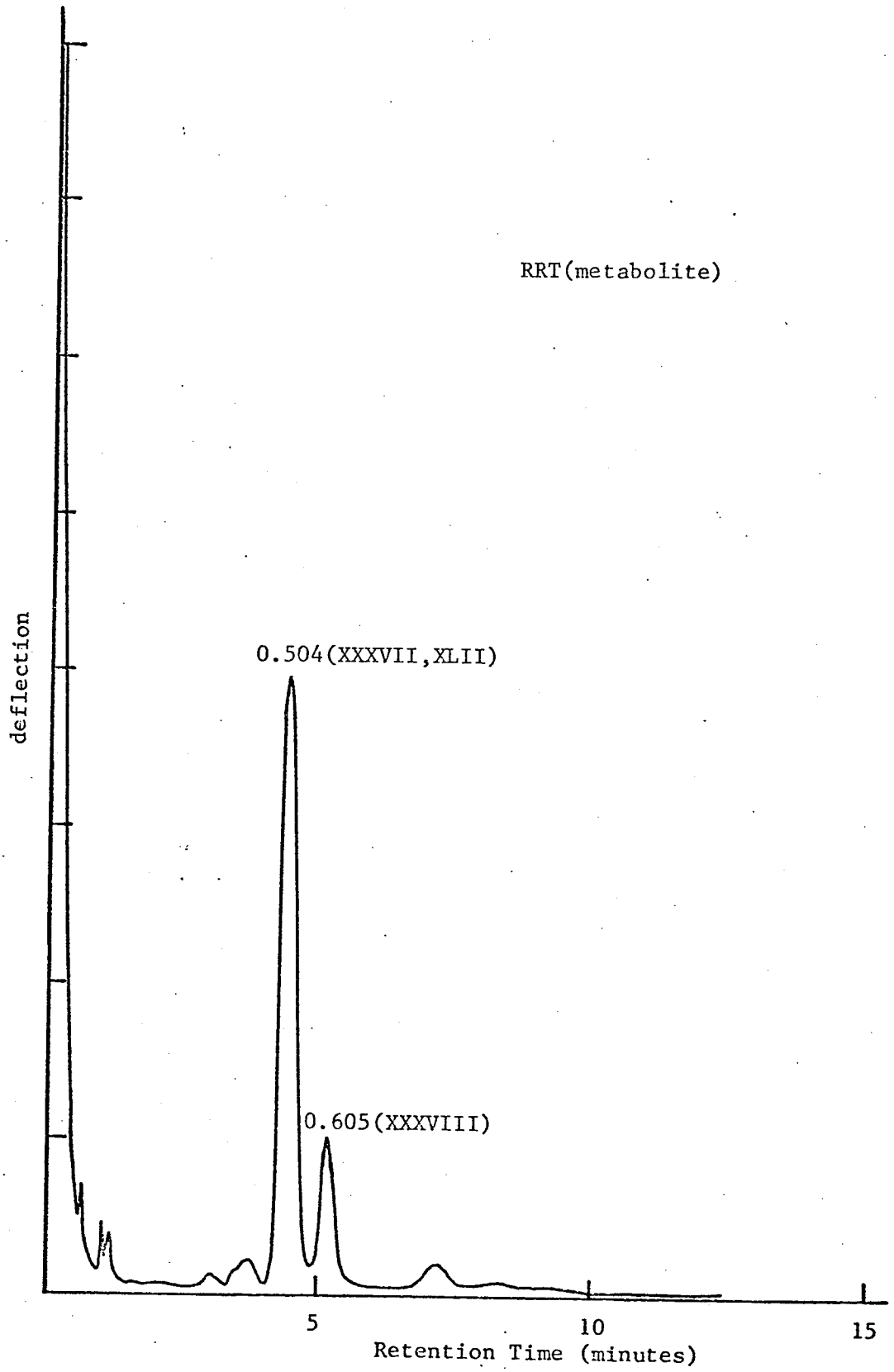


Fig. 27. GLC analysis of the non-ketonic fraction of the total urinary extract, after oral administration of II.

2α,3α-Cyclopropano-5α-androstane-4α,17α-diol(XXXVII)

Crystallization of the crude residue from methanol yielded XXXVII (249 mg), m.p. 215-218°. Further recrystallization gave an analytical sample m.p. 222-224°; R_f, 0;495(System Ia); RRT, 0.504; i.r. ν max 3285 and 3340(OH), 3030, 3090(cyclopropyl C-H) cm^{-1}; p.m.r., δ: 0.086(m, 1H, cyclopropyl proton), 0.63(s, C-18), 0.82(s, C-19), 3.72(d, H=6Hz, 17β-H), 3.85(q, J=4 and 6Hz, 4β-H) ppm; m.s., m/e: 304(M^+).

Anal., Found: C, 79.20; H, 10.62. $C_{20}H_{32}O_2$ requires C, 78.90; H, 10.59.

2α,3α-Cyclopropano-5α-androstane-4,17-dione(XL)

Jones oxidation of XXXVII gave the 4,17-dione(XL), m.p. 223-224° from methanol; R_f, 0.617(System Ia); RRT, 1.577; i.r. ν max 3060, 3080(cyclopropyl C-H), 1682(C=O, conjugated with a cyclopropane ring), 1739(5-membered ring C=O) cm^{-1}; p.m.r., δ: 0.85(s, C-18 and C-19) ppm; m.s., m/e: 300(M^+). Anal. Found: C, 80.13; H, 9.50. $C_{20}H_{28}O_2$ requires C, 79.96; H, 9.39.

17β-Hydroxy-2α,3α-cyclopropano-5α-androstan-4-one

To a solution of the 4.17-dione (20 mg) in ethanol (2 ml) was added sodium borohydride (24 mg) in ethanol (1 ml). The mixture was stirred at room-temperature for 1 hr. Dilution with excess dilute HCl and dichloromethane extraction yielded 17β-hydroxy-2α,3α-cyclopropano-5α-androstan-4-one (10.7 mg), m.p. 213-214°; R_f, 0.558(System Ia); RRT, 1.16, i.r. ν max 3481 (OH), 1686(C=O, conjugated with cyclopropane ring) cm^{-1}; m.s., m/e: 302(M^+).

2α,3α-Cyclopropano-5α-androstan-4β,17β-diol(XLI)

Further reduction of 17β-hydroxy-2α,3α-cyclopropano-5α-androstan-4-one and its mother liquor (18.6 mg) with excess $NaBH_4$ (54 mg) for 5 hrs. at room-temperature gave 2α,3α-cyclopropane-5α-androstane-4β,17β-diol(XLI, 12mg), m.p. 158-160°; R_f, 0.65(System Ia); RRT, 0.571; i.r. ν max 3060(cyclopropyl C-H str.) 3400 (OH) cm^{-1}; p.m.r., δ: -0.173(q, J=5Hz, 1H) and 0.469(sextet, J=4 and 6Hz, 1H), cyclopropyl protons, 0.659(s, C-18), 0.991(s, C-19), 3.602(t, J=8Hz, 17α-H), 3.91(d, J=4Hz, 4α-H) ppm; m.s., m/e: 304(M^+).
Anal. Found: C, 78.89; H, 10.64. $C_{20}H_{32}O_2$ requires C, 78.90; H, 10.59.

Chromatography of the crude residue:

The remaining crude urinary extract (1.28 g) was dissolved in 75% benzene/petroleum ether and chromatographed on alumina (45 g). A total of 145 fractions of 50 ml each were eluted with solvents of increasing polarity. Fractions of similar components (by GLC) were combined in 12 fractions. Table 6 shows the fractions obtained from column chromatography of the crude residue with RRT of the metabolites present.

Table 7. Fractions of column chromatography (Al_2O_3) and RRT of metabolites dosed with 17β-hydroxy-2α,3α-cyclopropano-5α-androstane

Column fractions	Weight (mg)	Solvent eluted	RRT*	Metabolite identified
1∿3	20	75% benzene/P.E.	0.350, 0.440	II
4∿9	245	75% benzene/P.E.	0.350, 0.440, 0.826	XXXIX
10∿20	153	87.5% benzene/P.E.	0.440, 0.605, 0.708	
			0.826	-------
21∿30	116	95% benzene/P.E.	0.605, 0.708	XXXVIII
31∿73	156	0∿5% ether/benzene	0.504, 0.605, 0.708	XXXVIII
74∿92	71	5∿25% ether/benzene	0.504, 0.605, 0.708	XLVI, XLII
93∿115	76	25∿100% ether/benzene	0.504, 0.605	
116∿123	15	ether	0.504, 0.605	XXXVII
124∿126	123	5% MeOH/ether	0.504, 0.605	-------
127∿132	58	5% MeOH/ether	0.866, 0.942	
133∿140	122	10∿20% MeOH/ether	0.866, 0.942	triol
141∿145	140	HOAc: MeOH: ether (5 : 10 : 85)	0.866, 0.942	
Total	1295			

*major peaks are underlined

4α-Hydroxy-2α,3α-cyclopropano-5α-androstan-17-one (XXXIX)

Elution with 75% benzene/petroleum ether yielded fractions (245 mg) showing mainly one GLC peak (RRT, 0.826). Repeated recrystallization from methanol gave an analytical sample (34 mg); m.p. 196-7°; Rf, 0.492 (System Ia); RRT, 0.826; i.r. ν max. 3560 (free OH), 3445 (bonded OH) 3030 and 3075 (cyclopropyl C-H) 1728 (C=O, 5-membered ring, shifted to 1745 in CCl_4) cm^{-1}; p.m.r. δ: 0.10(q, J=5Hz, 1H) and 0.64(m, 1H) cyclopropyl protons, 0.84(s, C-18 and C-19) 3.86(q, J=4 and 6Hz, 4β-H) ppm; m.s., m/e: 302(M^+).

Anal. Found: C, 79.52 H; 10.08. $C_{20}H_{30}O_2$ requires C, 79.42; H, 10.00.

Jones oxidation of XXXIX gave identical 4,17-dione (XL) by m.p., m.m.p., i.r., p.m.r., and m.s.

2α,3α-Cyclopropano-5α-androstane-4α,17β-diol (XXXVIII)

Elution with 95% benzene/petroleum ether (116 mg) showed a major peak (RRT 0.605). Repeated crystallization from methanol yielded XXXVIII, m.p. 215-215.5°; R_f, 0.534(System Ia); RRT, 0.605; ν max. 3450 (OH), 3020 and 3080 (cyclopropyl C-H) cm^{-1}; p.m.r., δ: 0.09(q, J=5Hz, 1H) and 0.63(m, 1H, cyclopropyl protons), 0.72(C-18), 0.82(s, C-19), 3.61(t, J=8Hz, 17α-H), 3.85(q, J=4 and 6Hz, 4β-H) ppm; m.s., m/e: 304(M^+).

Anal. Found: C, 79.21; H, 10.66. $C_{20}H_{32}O_2$ requires C, 78.90; H, 10.59.

Jones oxidation yielded identical 4,17-dione (XL) by m.p., m.m.p., i.r., and m.s.

17β-Hydroxy-3β-methyl-5α-androstan-2-one (XLVI) and 2α,3α-cyclopropano-5α-androstane-6α,17β-diol (XLII)

Fractions eluting with benzene to 25% ether/benzene (227 mg) were

rechromatographed. Elution with benzene (35 mg) yielded the 4α,17β-diol (XXXVIII).

Fractions eluting with 5% ether/benzene (57 mg) was separated into a ketonic and non-ketonic fraction with Girard T-reagent. The non-ketonic (32 mg) material further yielded the 4α,17β-diol (XXXVIII). The ketonic material gave 17β-hydroxy-3β-methyl-5α-androstan-2-one (XLVI), m.p. 145°; Rf, 0.595(System Ia); RRT, 0.708; i.r. (CCl_4) ν max. 3630 (OH), 1712 (C=O, 6-membered ring) cm^{-1}; p.m.r., δ: 0.725 (s, C-18 and C-19), 0.995(d, J=7Hz, 3β-CH_3), 3.63(t, J=8Hz, 17α-H) ppm; m.s., m/e: 304 (M^+).

Elution with 50% ether/benzene yielded a residue (92 mg) which on recrystallization from methanol yielded 2α,3α-cyclopropano-5α-androstane-6α,17β-diol (XLII) (43 mg), m.p. 209-210°; Rf, 0.467 (System Ia); RRT 0.504, i.r. ν max. 3350 (OH), 3070 (cyclopropyl C-H) cm^{-1}; p.m.r. δ: -0.18(q, J=5Hz, 1H) and 0.57(sextet, J=4Hz, 1H, cyclopropyl protons); 0.72(s, C-18); 0.81(s, C-19), 3.37(sextet, J=5 and 7 Hz, 6β-H), 3.62(t, J=8Hz, 17α-H) ppm; m.s., m/e: 304 (M^+).
Anal. Found: C, 79.14; H, 10.66. $C_{20}H_{32}O_2$ requires C, 78.90, H, 10.59.

2α,3α-Cyclopropano-5α-androstane-6,17-dione (IX)

Jones oxidation of XLII (10 mg) yielded the 6,17-dione (XLIII) (8.5 mg), m.p. 170-171° from methanol; R_f 0.667(System Ia); RRT, 1.198; i.r. ν max. 3030, 3070 (cyclopropyl C-H), 1710 (C=O, 6-membered ring), 1743 (C=O, 5-membered ring) cm^{-1}; p.m.r. δ: 0.22(q, J=5Hz, 1H) and 0.65(m, 1H, cyclopropyl protons), 0.78(s, C-19), 0.87(s, C-18) ppm; m.s., m/e: 300 (M^+).

Anal. Found: C, 80.15; H, 9.29. $C_{20}H_{28}O_2$ requires C, 79.96; H, 9.39.

2α,3α-Cyclopropano-5α-androstane-4α,17α-diol (XXXVII)

Elution with ether yielded 15 mg. which on recrystallization from methanol yielded XXXVII (5.6 mg) identical (m.p., m.m.p., i.r., m.s. and RRT) with the major substance.

Triol

Fractions eluted with 5-20% methanol/ether (140 mg) and 5% HOAc/10% MeOH/85% Et_2O (180 mg) which showed a broad peak on GLC (RRT, ∿0.980) were combined and rechromatographed. No pure materials were isolated. Fractions eluted with 5-20% methanol/ether were separated into a ketonic and non-ketonic fractions by Girard T-reagent. The non-ketonic fraction yield a triol mixture, m.p. 246-250°; Rf, 0.18 (System Ia); RRT, 0.866 and 0.942; i.r. ν max. 3400 (OH), 3070 (cyclopropyl C-H) cm^{-1}: m.s., m/e 320 (M^+), 305(M^+-CH_3), 302(M^+-H_2O). Ketonic material was a diolone, i.r. ($CHCl_3$) of impure substance, 3610 (OH), 1703 (C=O, 6-membered ring) cm^{-1}; m.s., m/e: 318 (M^+).

Oxidation with Jones reagent gave a non-crystalline product, i.r. ν max. 1735 (broad) 1684, cm^{-1}; m.s. m/e: 316 (M^+).

Isolation of neutral steroids (acid hydrolysis):

Urine (2080 ml) was collected over nine days from four rabbits each dosed four times at two day intervals with 17β-hydroxy-2α,3α-cyclopropano-5α-androstane (0.6 g) in a slurry of proplyene glycol (10 ml) by oral administration. Urine was collected under a layer of toluene and stored daily at -5°. Urine was adjusted to pH 1 with conc. HCl and heated to reflux for 30 min. Extraction with ether yielded the crude neutral extract (2 g). A control experiment yielded a crude extract (267 mg) from urine (2780 ml) collected from animals dosed as above with propylene glycol (10 ml).

A-Homo-5α-androst-4-ene-2ξ,17α-diol (L)

A portion of total crude urinary extract from acid hydrolysis (1.25 g) was seperated into non-ketonic (900 mg) and ketonic fractions (300 mg) by Girard T-reagent. From the non-ketonic fraction, a major substance was crystallized from acetone (165 mg) which on repeated recrystallization from methanol yielded A-homo-5α-androst-4-ene-2β, 17α-diol (L), m.p. 220°, R_f, 0.258(System Ia); RRT, 0.449; i.r. ν max. 3400 (OH), 3030 (olefinic C-H) cm^{-1}; p.m.r., δ: 0.67(s, C-18), 0.86 (s, C-19), 3.64(m, $W_{\frac{1}{2}}$=22Hz, 2ξ-H), 3.75(d, J=6Hz, 17β-H), 5.23(q, 1H) and 5.68(m, 1H), (olefinic protons) ppm.; m.s., m/e: 304 (M^+). Anal. Found: C, 78.91; H, 10.51. $C_{20}H_{32}O_2$ requires C, 78.90; H, 10.59.

A-homo-5α-androst-3- and 4-ene-2,17-dione (LIa and LIb)

Jones oxidation of A-homo-5α-androst-4-ene-2β,17α-diol (XLVIII, 40 mg) yielded noncrystalline mixture (37 mg) of A-homo-5α-androstan-3- and 4-ene-2,17-dione; i.r. (CCl_4), 3030 (olefinic C-H str.), 1744 (5-membered ring C=0), 1708 (7-membered β,γ-unsaturated C=0), 1688 (α,β-unsaturated C=0) cm^{-1}; p.m.r., δ: 0.864, s and 0.914, s, (C-18 and C-19, LIb), 0.889, s, and 0.923, s, (C-18 and C-19, LIa), 5.561 (m, 1H) and 5.734 (m, 1H, olefinic protons) ppm; m.s., m/e: 300 (M^+).

Treatment of the above product (16 mg) with excess 2% methanolic KOH at reflux for 1½ hrs. yielded a second noncrystalline product; i.r. (CCl_4), 1745(C=0, 5-membered ring), 1712(7-membered β,γ-unsaturated C=0).

C. Metabolism of 17β-hydroxy-2α-methyl-5α-androstan-3-one (dromostanolone)

Preparation of dromostanolone

Dromostanolone propionate (Lilly Lab. Lot No. B01279) (10 g) was refluxed with 2% (0.36N) methanolic KOH (200 ml) for 1 hour. The reaction was concentrated at reduced pressure to half volume, cooled, and water added slowly until crystallization was complete. The reaction mixture was cooled in an ice-bath, filtered, and thoroughly washed with cold MeOH:H_2O (1:1) to yield dromostanolone (7.22 g), m.p. 151-151.5°, (lit.[121] m.p. 151-153°) i.r. ν max 3470 (OH), 1701 (6-membered ring C=0), p.m.r., δ: 0.757(s, C-18), 1.072(s, C-19), 1.000(d, 2α-CH_3), 3.64(t, J=8Hz, 17α-H); ppm; m.s., m/e: 304 (M^+). steroid was chromatographically pure (TLC, GLC).

2α-Methyl-5α-androstane-3β,17β-diol (LVI)

Dromostanolone (III, 775 mg) was reduced by sodium borohydride to yield a crude product (661 mg), m.p. 165-170°. Recrystallization from methanol yielded LVI, m.p. 174-175° (lit.[122] m.p. 175-177°), R_f, 0.467(System Ia), RRT, 0.499, i.r. 3450 (OH) cm^{-1}; m.s., m/e: 306(M^+).

2α-Methyl-5α-androstane-3,17-dione (XLIX)

Dromostanolone (III, 55 mg) was dissolved in acetone (2 ml) and oxidized with excess Jones reagent (0.5 ml) to give a crude product (46 mg). Recrystallization from methanol yielded XLIX, m.p. 152-153° (lit.[123] m.p. 152-154°). R_f, 0.692(System Ia); RRT, 1.048; i.r. (CCl_4) 1706(6-membered ring C=0), 1744(5-membered ring C=0); m.s., m/e: 302(M^+).

Administration of dromostanolone

Three rabbits were each dosed (0.7 g) three times at two-day intervals with dromostanolone in propylene glycol (10 ml). Urine (3850 ml) was collected for a total of seven days. Urine collected for a further 48 hours (1200 ml) gave a crude residue (53 mg) which showed no metabolite peaks on GLC. Blank urine (3080 ml) from rabbits dosed with propylene glycol (10 ml) as above was collected similarly prior to dosing (183 mg).

Isolation of neutral steroids

The pooled urine (3800 ml) was hydrolyzed with β-glucuronidase and extracted with ether to give a crude neutral urinary extract (1.84 g).

Oxidation of crude neutral steroids

A portion (100 mg) of the total crude urinary extract on oxidation with Jones reagent gave a crystalline substance (77 mg). TLC in system Ia and Ib showed one major spot with identical R_f (0.692 System Ia) with 2α-methyl-5α-androstane-3,17-dione and a minor spot (R_f, 0.216 System Ia). GLC showed one major peak (RRT, 1.048) identical to 2α-methyl-5α-androstane-3,17-dione (86.8%) and minor peaks (RRT, 1.215, 1.326, 1.580) as shown in Fig. 28.

Recrystallization of the total oxidized product from MeOH gave 2α-methyl-5α-androstane-3,17-dione XLIX, m.p. 150-151° (lit.[123] m.p. 152-154°), i.r. ν max. 1743(5-membered ring C=0), 1706(6-membered ring C=0) cm^{-1}; m.s., m/e: 302(M^+). Mixed melting point with an authentic sample was not depressed. Comparison of i.r. and m.s. spectra showed them to be identical.

The mother liquor indicated the presence of trione, m/e 316(M^+)

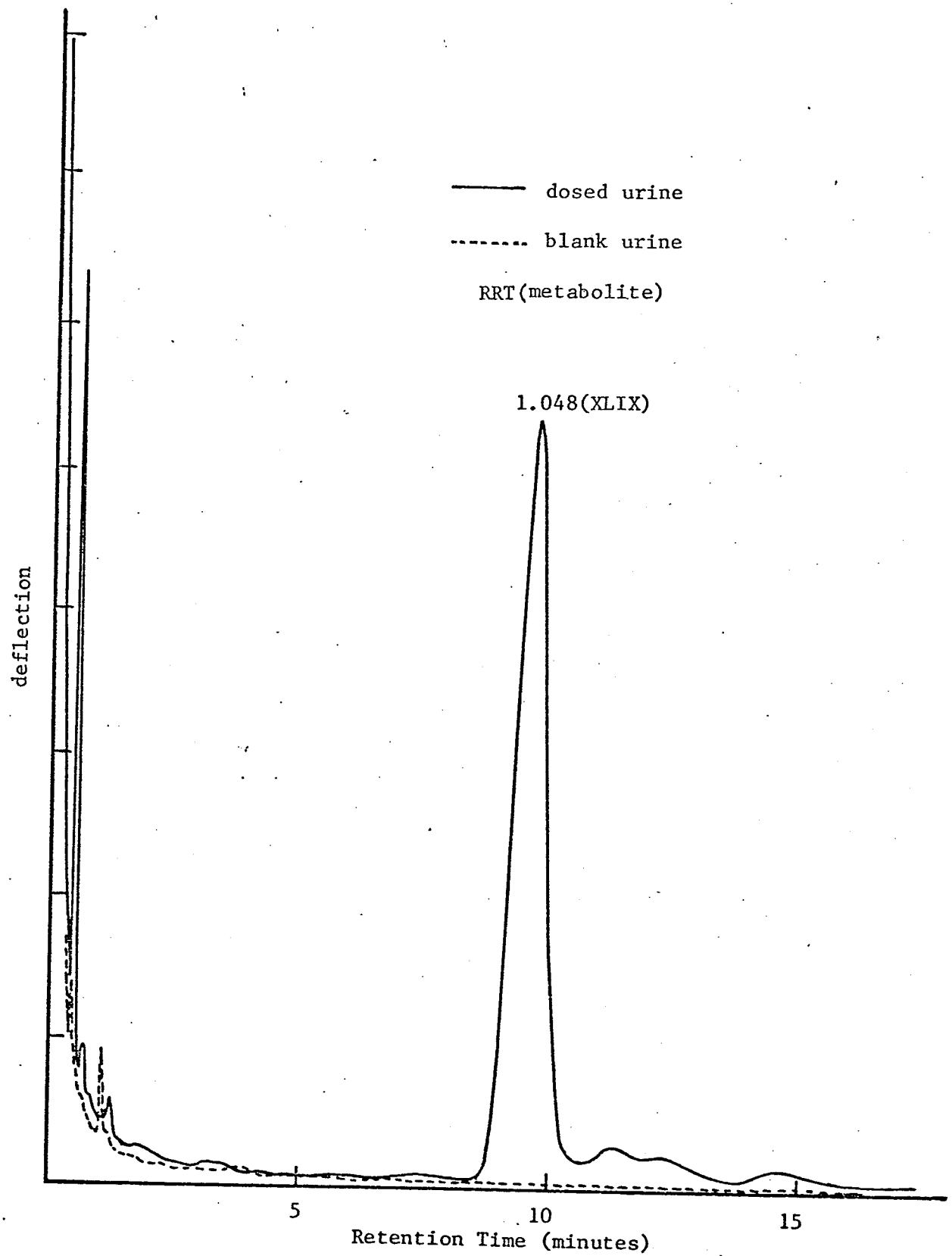


Fig. 28. GLC analysis of the oxidation product of the total urinary extract, after oral administration of III.

and dionol, m/e: 318(M^+).

GLC/m.s. of the major peak gave a m.s. spectrum identical with the 3,17-dione.

Separation of ketonic and non-ketonic materials:

The remaining crude material (1.74 g) was treated with Girard T-reagent[86] to give a crude non-ketonic fractions (1.050 g) and a ketonic fraction (611 mg). GLC of the crude extract, ketonic and non-ketonic material is given in figs. 25, 26 and 27.

Ketonic fraction;

3α-Hydroxy-2α-methyl-5α-androstan-17-one (LIII)

The ketonic fraction contained mainly one substance (TLC, GLC). A portion (443 mg) of the crude ketonic material was chromatographed on ethyl acetate washed alumina (8 g). From the fractions eluted with benzene (253 mg) a crystalline substance was obtained, which on recrystallization from methanol gave 3α-hydroxy-2α-methyl-5α-androstan-17-one (LIII, 32 mg), m.p. 195-196°; R_f, 0.558(System Ia); RRT, 0.518; i.r. ν max 3500 (OH), 1730 (5-membered ring C=O, H-bonded); p.m.r., δ: 0.80(s, C-19), 0.85(s, C-18), 0.90(d, J=7Hz, 2α-methyl), 3.77(m, $W_{\frac{1}{2}}$=7Hz, 3β-H) ppm; m.s., m/e: 304(M^+).

Anal. Found: C, 78.94; H, 10.52. $C_{20}H_{32}O_2$ requires C, 78.90; H, 10.59.

The mass spectra of more polar fraction eluted with ether and methanol indicated the presence of diolone (m/e, 320) and dionol (m/e,318) substances. Rechromatography of these fractions on ethyl acetate treated alumina of these fractions did not separate these materials in pure forms.

2α-Methyl-5α-androstane-3,17-dione (XLIX)

Oxidation of 2α-methyl-5α-androstane-3α-ol-17-one (LIII) with Jones reagent gave 2α-methyl-5α-androstane-3,17-dione (XLIX), m.p. 152.5-153.5°, with identical i.r. and m.s. spectral data to an authentic sample. Mixed melting point was not depressed.

Non-ketonic fraction;

The total crude non-ketonic material (982 mg) was dissolved in 20% petroleum ether/benzene and chromatographed on alumina (34 g). A total of 170 fractions of 50 ml each were eluted with solvents of increasing polarity. Fractions of similar components (by GLC) were combined and from the fractions containing mainly one substance, the following metabolites were isolated by repeated crystallization.

2α-Methyl-5α-androstane-3α,17α-diol (LIV)

From fraction eluted with ether and 2.5% MeOH/ether which contained only one substance (TLC, GLC) on recrystallization from MeOH yielded LIV (34 mg); m.p. 151°; R_f 0.442(System Ia), RRT 0.292; i.r. ν max 3430 (OH) cm^{-1}; p.m.r. δ: 0.643(s, C-18), 0.795(s, C-19), 0.927(d, J=7Hz, 2α-methyl), 3.686(d, 1H, J=6Hz, 17β-H), 3.754(m, $W_{\frac{1}{2}}$= 7Hz, 1H, 3β-H), ppm; m.s., m/e: 306(M^+).

Anal. Found: C, 78.50; H, 11.21, $C_{20}H_{34}O_2$ requires C, 78.38; H, 11.18.

2α-Methyl-5α-androstane-3β,17α-diol (LV)

Fractions eluted with 10∿25% ether/benzene (43 mg) were recrystallized from MeOH to yield LV (18 mg), m.p. 215-216°; R_f, 0.458(System Ia); RRT, 0.419; i.r. ν max. 3230-3400 (OH) cm^{-1}; p.m.r., δ: 0.643(s, C-18), 0.823(s, C-19), 0.966(d, J=7Hz, 2α-methyl), 3.114 (sextet, $W_{\frac{1}{2}}$=27Hz, 1H, 3α-H), 3.710(d, J=6Hz, 1H, 17β-H) ppm; m.s., m/e: 306(M^+).

Anal. Found: C, 78.09; H, 11.12. $C_{20}H_{34}O_2$ requires C, 78.38; H, 11.18.

2α-Methyl-5α-androstane-3β,17β-diol (LVI)

Fractions eluted with 5% ether/benzene (7 mg) contained mainly one substance (TLC and GLC). The major substance was separated on preparatory plate (developed 3x in System Ib) and purified by filtering through ethyl acetate washed alumina in benzene to yield LVI. R_f, 0.467(System Ia); RRT, 0.499; i.r., 3450 (OH) cm^{-1}; m.s., m/e: 306(M^+). Comparison with an authentic sample showed them to be identical (R_f, RRT, i.r., m.s.).

2α-Methyl-5α-androstane-3α,15α,17α-triol (LVIIa)

Fractions eluted with 5% MeOH/ether (63 mg) showed the presence of mainly one peak (RRT 0.364). Recrystallization from methanol yielded LVIIa (8.2 mg), m.p. 215-216°; R_f 0.05 (System Ia); i.r. ν max 3520 and 3450 (OH) cm^{-1}; p.m.r., δ: 0.689(s, C-18), 0.811(s, C-19), 0.930 (d, J=7Hz, 2α-methyl), 3.648(d, J=6Hz, 1H, 17β-H), 3.759(m, $W_{\frac{1}{2}}$=7Hz, 1H, 3β-H), 4.014(sextet, 1H, 15β-H) ppm; m.s., m/e: 304(M^+-H_2O). Anal. Found: C, 72.97; H, 10.48. $C_{20}H_{34}O_3 \cdot \frac{1}{2}H_2O$ requires C, 72.47; H, 10.63.

2α-Methyl-5α-androstane-3α-15α,17α-triol triacetate (LVIIb)

Acetylation of LVIIa gave a non-crystalline product LVIIb, R_f, 0.375 (System Ib); p.m.r., δ: 0.802(s, C-18), 0.818(s, C-19), 0.834(d, J=7Hz, 2α-Me), 2.007, 2.045, 2.068(3H-singlets, OAc), 4.754 (d, J=6Hz, 1H, 17α-OAc), 4.923(m, 2H, 3α-OAc overlapped with 15α-OAc)ppm; m.s., m/e: 388(M^+-HOAc).

2α-Methyl-5α-androstan-3,15,17-trione

Jones oxidation of LVIIa yielded an impure, non-crystalline substance, m.s., m/e: 316(M^+).

2α-Hydroxymethyl-5α-androstane-3α,16α,17α-triol (LIXa)

Fractions eluted with 25-100% methanol/ether and HOAc:MeOH: Et_2O(5:10:85) were combined (172 mg). These fractions showed three peaks on GLC(RRT; 0.553, 0.660 and 0.835) which on recrystallization from methanol yielded a crystalline substance (RRT, 0.835), LIXa (18 mg), m.p. 248-250°; R_f, 0.025(System Ia, developed 3x), i.r., ν max 3100-3500 (OH); m.s., m/e: 338(M^+).

2α-Methyl-5α-androstane-3α,16α,17α-triol (LVIIIa)

The mother liquor from LIXa was applied to thick-layer preparatory plate, developed three times (System Ia) and separated into three bands. From the least polar band (R_f, 0.475, System Ia, developed 3x) a crystalline substance was obtained which on re-crystallization from methanol gave LVIIIa (2 mg), m.p. 240°; RRT, 0.553; p.m.r., δ: 0.709(s, C-18), 0.811(s, C-19), 0.955(d, J=7Hz, 2α-methyl), 3.636(d, J=6Hz, 17β-H), 3.777(m, $W_{\frac{1}{2}}$=7Hz, 3β-H), 4.443 (m, 16β-H) ppm; m.s., m/e: 322(M^+).

2α-Methyl-5α-androstane-3α,16α,17α-triol triacetate (LVIIIb)

Acetylation of the remaining bands from the preparatory plates (see LVIIIa) and further separation on a thick-layer chromatogram gave a non-crystalline product (LVIIIb) from the least polar band corresponding to the acetate of LVIIIa; R_f, 0.483(System Ib, developed 2x); p.m.r., δ: 0.798(s, C-18), 0.818(s, C-19), 0.841(d, J=7Hz, 2α-methyl), 2.000, 2.057, 2.089 (3H-singlets, OAc), 4.932(m, $W_{\frac{1}{2}}$=7Hz, 3α-OAc), 5.000(d, J=6Hz, 17α-OAc), 5.364(m, 16α-OAc) ppm; m.s., m/e: 388(M^+-HOAc).

2α-Acetoxymethyl-5α-androstane-3α,16α,17α-triol triacetate (LIXb)

The second band from the thick-layer separation of the acetylated material (see LVIIIb) yielded a non-crystalline product (LIXb), R_f, 0.275(System Ib, developed 2x); p.m.r., δ: 0.820(s, C-18), 0.820(s, C-19), 2.002, 2.030, 2.045, 2.086(4H-singlets, OAc), 3.814 and 3.960(m, 2H, $-CH_2-$OAc at C-2), 5.000(d, J=6Hz, 17α-OAc), 5.068(m, $W_{\frac{1}{2}}$=7Hz, 3α-OAc), 5.361(m, 16α-OAc) ppm; m.s., m/e: 446(M^+-HOAc).

V. APPENDIX

APPENDIX A

Androgenic-Myotrophic Assay of 5α-Androstan-3-one(I), 17β-Hydroxy-2α,3α-cyclopropano-5α-androstane(II) and 17β-Hydroxy-2α-methyl-5α-androstan-3-one(III).

Compd.	Ventral prostate	Seminal vesicle	Levator ani	Std.	Q	Adm. Rte.	Ref.
T(a)	190	112	80		1	SC	*
Control(a)	24	15	31			SC	*
I(a)	25	15	29			SC	*
II(b)	30	30	100	TP	3	SC	**
III(b)	24	25	62	T	3	SC	***
		40	130	T	3	SC	****

(a) weight(mg)
(b) biological activity indices
T: Testosterone
TP: Testosterone propionate
SC: subcutaneous
Q: The average anabolic/androgenic ratio(quotient)

Ref. * by Endocrine Laboratories of Madison, Inc., 679 Jonathan Dr., Madison, Wisconsin, 53713.
** M.E.Wolff, W.Ho and R.Kwok, J. Med. Chem., 7, 577 (1964).
*** F.A.Kincl and R.I.Dorfamn, Steroids 3, 109 (1964).
**** F.A.Kincl, Methods Hormone Res., 4, 21 (1965).

APPENDIX B

Comparison of the calculated (C) and found (F) values of the

(i) p.m.r. chemical shifts of C-19 and C-18 protons

(ii) difference between C-19 and C-18 chemical shifts.

Metabolite		(i) ppm C-19	(i) ppm C-18	(ii) ppm $\Delta(C_{19}-C_{18})$
5α-androstane-3α,16α-diol	(C)	0.77	0.71	0.06
	(F)	0.805	0.725	0.08
5α-androstane-3β,16α-diol	(C)	0.80	0.71	0.09
	(F)	0.82	0.72	0.10
5α-androstane-3β,17α-diol	(C)	0.79	0.64	0.15
	(F)	0.815	0.655	0.16
3α-hydroxy-5α-androstan-16-one	(C)	0.82	0.87	0.05
	(F)	0.83	0.89	0.06
3β-hydroxy-5α-androstan-16-one	(C)	0.85	0.87	0.02
	(F)	0.865	0.89	0.025
2α,3α-cyclopropano-5α-androstane-4α,17α-diol	(C)	0.81	0.62	0.19
	(F)	0.82	0.64	0.18
2α,3α-cyclopropano-5α-androstane-4α,17β-diol	(C)	0.784	0.673	0.111
	(F)	0.814	0.72	0.094
4α-hydroxy-2α,3α-cyclopropano-5α-androstan-17-one	(C)	0.84	0.83	0.01
	(F)	0.845	0.836	0.01
2α,3α-cyclopropano-5α-androstane-4,17-dione	(C)	0.78	0.81	0.03
	(F)	0.85	0.85	0.00

APPENDIX (cont'd)

Metabolite		(i) ppm C-19	(i) ppm C-18	(ii) ppm $\Delta(C_{19}-C_{18})$
2α,3α-cyclopropano-5α-androstane-6α,17β-diol	(C)	0.804	0.703	0.101
	(F)	0.814	0.72	0.094
2α,3α-cyclopropano-5α-androstane-6,17-dione	(C)	0.77	0.85	0.08
	(F)	0.776	0.866	0.09
2α,3α-cyclopropano-5α-androstane-4β,17β-diol	(C)	1.02	0.66	0.36
	(F)	0.991	0.659	0.332
3α-hydroxy-2α-methyl-5α-androstan-17-one	(C)	0.805	0.853	-0.048
	(F)	0.80	0.85	-0.05
2α-methyl-5α-androstane-3α,17α-diol	(C)	0.77	0.63	0.14
	(F)	0.795	0.643	0.152
2α-methyl-5α-androstane-3β,17α-diol	(C)	0.80	0.63	0.17
	(F)	0.823	0.643	0.18
2α-methyl-5α-androstane-3α, 15α,17α-triol	(C)	0.805	0.673	0.132
	(F)	0.811	0.689	0.122
2α-methyl-5α-androstane-3α, 15α,17α-triol triacetate	(C)	0.821	0.792	0.029
	(F)	0.818	0.802	0.016
2α-methyl-5α-androstane-3α, 16α,17α-triol	(C)	0.785	0.653	0.132
	(F)	0.811	0.709	0.102
2α-methyl-5α-androstane-3α, 16α,17α-triol triacetate	(C)	0.808	0.800	0.008
	(F)	0.818	0.798	0.02
2α-hydroxymethyl-5α-androstane-3α,16α,17α-triol triacetate	(C)	0.808	0.800	0.008
	(F)	0.820	0.820	0.00

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