STUDIES ON THE ANABOLIC STEROIDS METHANDROSTENOLONE AND OXYMETHOLONE AND THEIR METABOLITES

by

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A Thesis presented for the Degree of Doctor of Philosophy

University of Edinburgh September, 1972.

Acknowledgements

The author would like to express her gratitude to Dr. P.J. Sykes for his guidance and help during this work, and to Professor J.I.G. Cadogan for the provision of technical and library facilities. Thanks are also due to Dr. R.A. Harkness of the Department of Paediatric Biochemistry, Royal Hospital for Sick Children, Edinburgh for the provision of metabolic and drug samples and for helpful discussion. The award of a maintenance grant from the Science Research Council is gratefully acknowledged.

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Summary

The principal object of the work described in this thesis has been the synthesis of compounds as standards for comparison with the metabolites of the anabolic steroid drugs methandrostenolone and oxymetholone.

Methandrostenolone is metabolised to two major metabolites, one of which has been previously identified as 6β-hydroxymethandrostenolone. The major metabolite of the drug in normal man has now been identified as 17-epi-methandrostenolone, the unambiguous synthesis of which is described. Prior to comparison of the previously unknown metabolite and 17-epimethandrostenolone the properties of the latter compound were extensively compared with those of its 17-epimer. Mass spectrometry of the free steroid and gas-liquid chromatography of the trimethylsilyl derivative were among the methods used to identify the metabolite.

Although urine extracts were examined unsuccessfully for evidence of methandrostenolone sulphate as a precursor of 17-epimethandrostenolone the chemical synthesis of sulphate and sulphonate derivatives of the tertiary 17β-hydroxy-17-methyl function was attempted. The products were unstable and were readily hydrolysed with retention of configuration but their mass spectra were similar to that of 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one, the product of the sulphuric acid-catalysed dehydration of methandrostenolone. The C-17 epimerisation of the drug could not be achieved by chemical means.

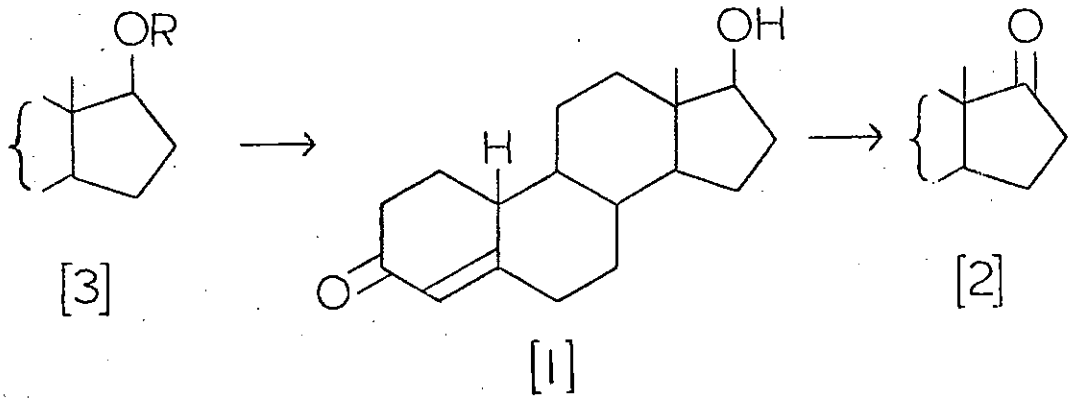
The previously unstudied drug oxymetholone is metabolised by normal man to two major metabolites which are excreted as conjugates of glucuronic acid. High temperature catalytic reduction of the drug and its metabolites gave only unsubstituted 17-methylandrostanes. Consequently, it was not known whether the C-2' carbon atom of oxymetholone was retained in the metabolites. The four 17α-methyl-5α-androstane-2,3,17-triols were synthesised and their mass spectra compared with those of the metabolites. None of the triols corresponded to either of the metabolites, which were tentatively identified by mass spectrometry and thin layer chromatography as 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol. The latter compounds were synthesised by the reductions of oxymetholone with a variety of metal hydrides.

The enzymatic reductions of 5β-androstane-3,17-dione, oxymetholone and methandrostenolone with horse liver alcohol dehydrogenase were examined. A low yield of 3β-hydroxy-5β-androstan-17-one was obtained from the former compound and 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one was tentatively identified as a product of the enzymatic reduction of oxymetholone, which was partially degraded in the alkaline buffer system. Methandrostenolone was not transformed by the enzyme.

1. Introduction

Kockakian,[1] in 1935, first observed that the androgens, the male sex hormones, possessed the ability to stimulate the synthesis of cellular protein, associated with the secondary male characteristic of muscle development. It was quickly appreciated that compounds with this property would provide valuable medications in the treatment of conditions involving abnormally low tissue protein content. However, the natural steroids which possess this anabolic effect were unacceptable for clinical use, particularly with women and children, because of the virilising androgenic effect.[2]

An assay of the relative anabolic and androgenic effects of a compound, the anabolic/androgenic ratio, was developed by Eisenberg and Gordan[3] and later modified by Hershberger *et al.*[4] Although there have since been many alterations to these original procedures and the methods are still open to criticism[5a] the assay provides a basis for the screening of possible anabolic agents. Examination of the natural androgens showed that almost all had an anabolic/androgenic ratio of *ca.* 1.0, so that if strongly androgenic they were also strongly anabolic but if only weak androgens the anabolic effect was weak. Thus began the search for steroids in which the effects were as widely separated as possible. According to Kruskemper[5b] "the androgenic effect differs from the anabolic effect only in its location and not in its essence, thus anabolic steroids which possess no androgenic character cannot exist". However, this opinion is not universally shared and anabolic steroids with no



[3] [1] [2]

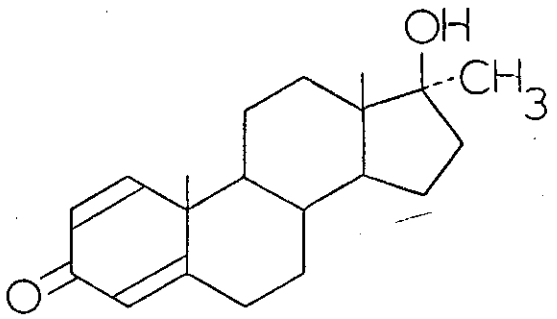
R = propionyl
sodium succinyl
phenyl propionyl
decanoyl
adamantoyl, etc.

androgenic action are still sought.[6]

The first anabolic steroid to be found[4] in which the androgenic activity was low enough to allow its clinical use was 19-nortestosterone[1]. However, a comparison of the doses required to produce a similar effect when the compound was administered orally and parenterally, respectively, revealed that the latter route required approximately one seventh of the oral dose.[7] This was explained by the fact that the orally administered drug was rapidly transported to the liver and deactivated by 17-oxidation[2].

Esterification of 19-nortestosterone led to a series of drugs[3] in which the relatively brief activity of the parenterally administered free steroid was significantly prolonged.[5c] Variation of the ester residue, particularly an increase in length, had the effect of increasing the duration of action and the anabolic/androgenic ratio.[2] The ester confers lipid solubility upon the steroid and consequently the longer the hydrocarbon chain the more lipid-soluble the steroid and the slower the diffusion into the blood stream where rapid hydrolysis to the active compound occurs.[8] The esters of 19-nortestosterone constitute a large group of the anabolic steroids in current clinical use.

The deactivation of testosterone and its derivatives by oxidation of the 17-hydroxyl group is prevented by the introduction of a 17α-alkyl substituent. This modification has resulted in a large group of orally active anabolic steroid drugs. 17β-Hydroxy-17-methylandrosta-1,4-dien-3-one(methandrostenolone)[4], and 17β-hydroxy-2-hydroxymethylene-17-



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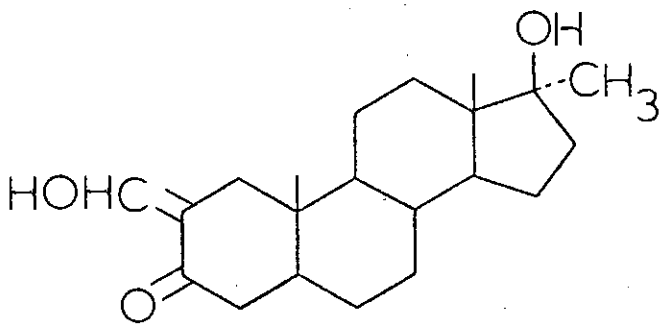

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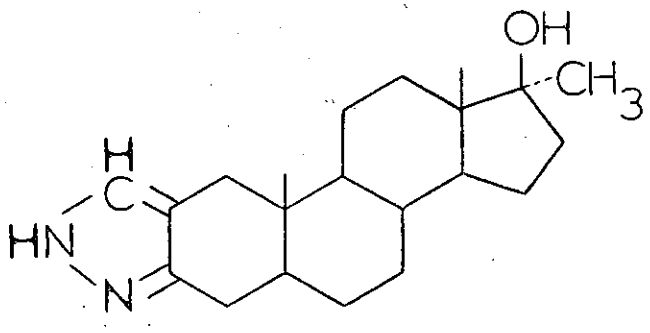


[6]

methyl-5α-androstan-3-one(oxymetholone)[5], an investigation of both of which is reported here, are members of this group.

A further group of compounds, less obviously related to the natural androgens, have been found to possess high anabolic/androgenic ratios and some are in clinical use.[2] Many of these steroids, such as 17β-hydroxy-17-methyl-5α-androstane-(3,2-c)-pyrazole[6] possess a heterocyclic ring fused to ring A at positions 2 and 3 and may be prepared from oxymetholone.

A wide variety of effects have been ascribed to the anabolic steroids, the chief of which are the positive influence on the protein content of the genital and extragenital organs, the inhibition of hypophyseal gonadotropin function, the anti-oestrogenic effect, the gestagenic effect and the intrauterine masculinising effect on female embryos.[5d] All anabolic steroids do not exhibit these properties to the same degree but any overall theory of their mechanism of action would need to encompass an explanation of the mechanisms by which they are responsible for each effect. The effect of the anabolic steroids on protein metabolism is the only one about which tentative statements may be made at present, protein synthesis being one of the better-studied areas of molecular biology. However correlations between the structure and degree of activity of anabolic steroids cannot be exact since they are based upon tests in which the use of different animal species, variations in steroid dosages, in methods of administration and in interpretation of data may lead to variable results.[5d]

Hormones affect metabolic processes by their ability

to determine the rates of reactions and consequently a particular hormone must have a high affinity for the system to be controlled. Steroid hormones, like many drugs, are bound to specific plasma proteins by non-covalent forces,[9] the significance of which is not yet fully understood. The ability of steroids to bind with plasma proteins is thought to be related to the requirement for efficient transport to the target organ.[10] As has already been illustrated with reference to 19-nortestosterone, a hormone must possess a structure such that it can survive for sufficient time to exercise its hormonal activity at the target organ before its deactivation by metabolic processes. The strength of binding with plasma proteins may be a factor in the rate of metabolic deactivation of structurally sensitive steroids as well as a control mechanism of hormonal activity at the target organ, only the free fraction being available to exert its effect.[10]

At the target organ the hormone may act on the surfaces of cells or intracellular phase boundaries thereby altering the permeability to substrates, it may interact with the enzyme systems or affect the synthesis of enzyme proteins from precursors.[5d] Anabolic steroids might promote a positive nitrogen balance by stimulation of protein synthesis, by deceleration of protein degradation or by a decrease in the conversion of amino acids to urea. Various studies have shown that protein degradation is not impeded but that anabolic steroids produce a definite enhancement of synthesis.[11,12] Examination of the mechanism of protein synthesis shows that anabolic steroids might intervene to exert their stimulatory

effect at many points in the process. An increase of the ribonucleic acid content of cells and of the activities of amino acid activating enzymes has been demonstrated but further work is necessary before the precise point at which anabolic steroids exert their effect can be determined.[5d]

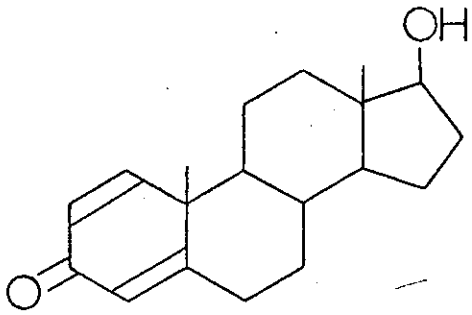
In order to exhibit anabolic activity steroids must thus be able to bind to plasma proteins for transport to the target organ, they must not be metabolised too rapidly and they must be capable of exerting an effect on the protein synthesising system. It is possible that each of these requirements may involve different structural features and that only those steroids which possess suitable combinations of these show activity.[10] This might explain why many anabolic steroids possess similar D-ring substituents but may have a comparatively large variety of substituents in the A-ring.

The first attempts to synthesise steroids with favourable anabolic/androgenic ratios involved relatively minor modifications to the natural steroids 17β-hydroxy-5α-androstan-3-one and testosterone, which are highly biologically active. In later work substituents such as alkyl groups and halogen atoms were introduced.[5d] Observations of the androgenic activity of 5α-androstan-17β-ol,[13] its 17α-methyl derivative[14] and 5α-androstane[15] and of the progestational activity of a series of 17-alkyl-3-deoxy-19-nortestosterones[16] dispelled the classical belief that functional groups at C-3 and C-17 were necessary for biological activity. Bowers *et al.*[10] synthesised a series of 3-deoxy compounds with at least one centre of unsaturation in the A-ring to facilitate the requirement for

protein binding. This work showed that a high electron density at either C-2 or C-3 or both in 17β-hydroxy-5α-androstane was a factor which strongly promoted anabolic activity. Klimstra et al.[17] studied the effect of the location of the oxygen atom in ring A on the anabolic activity of a series of androstane derivatives and found that 1-oxy substituted compounds were highly active.

Methandrostenolone was first prepared[18] in 1955 during a survey of the use of microorganisms which effected 1,2-dehydrogenation. Animal experiments showed that the compound had a high anabolic/androgenic ratio and strongly promoted nitrogen retention with relatively small hormonal side effects. As the drug Dianabol(Ciba) it has found wide application as an oral anabolic agent. The introduction of a 1,2-double bond into 17α-methyltestosterone resulted in increased biological activity, compared to the less active 5,6- and 6,7-dehydro compounds.[5d]

Recent work[19] on the X-ray crystallographic structure determination of 17β-hydroxyandrosta-1,4-dien-3-one[7], the first steroid containing the 1,4-dien-3-one system to be studied by this technique, is of particular interest with regard to the relationship between the structure and biological activity of methandrostenolone and other steroids which possess this A-ring. Measurements of the valence angles and bond lengths showed that the A-ring is bent towards the α-side of the molecule to a much greater extent than can be assessed from molecular models. This change in conformation of the A-ring has the effect of greatly altering the position of the



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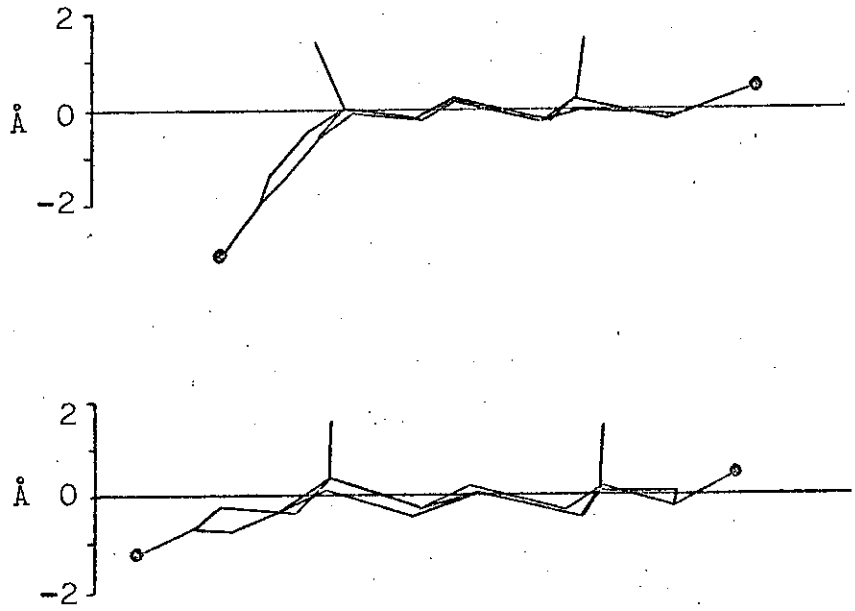
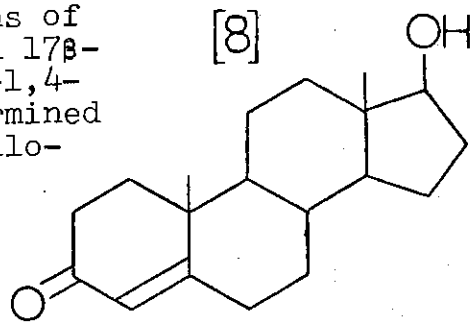


Figure I.

The conformations of testosterone and 17β-hydroxyandrosta-1,4-dien-3-one determined by X-ray crystallography.

[8]



19-methyl group with respect to the rest of the molecule. The angle between the planes containing C-19, C-10 and C-13, C-18 is 10.1° in 17β-hydroxyandrosta-1,4-dien-3-one[7] compared to 0.8° in testosterone[8], resulting in an increase of 0.5Å in the distance between C-18 and C-19 in the former compound.

In addition, the length of the 13-18 bond was found[19] to be 1.51Å, unusually short compared with the theoretical $C(sp^3)$-$C(sp^3)$ bond distance (1.526Å). The other three bonds to C-13 are all longer, giving an average bond length to C-13 of 1.54Å, only slightly longer than normal. The short 13-18 length was ascribed to the bowing of the steroid towards the α-side which relieves crowding around the methyl groups and to interaction of the angular methyl groups and the phenol of p-bromophenol used as a complexing agent to obtain the X-ray spectra.

The conformations of testosterone[8] and its 1-dehydro analogue[7] are shown in figure 1. Since the structure of methandrostenolone has not been studied by X-ray crystallography the effect of the additional 17α-methyl group upon the conformation cannot be assessed. However it is probable that the A-ring will be bent towards the α-side of the molecule as in 17β-hydroxyandrosta-1,4-dien-3-one.

Oxymetholone was synthesised[20] in 1959 as an intermediate in the preparation of 2α,17α-dimethyl-17-hydroxy-5α-androstan-3-one during a programme designed to study the effects of alkyl substituents on biological activity in the androstane and 17α-alkylandrostane series. It was found to

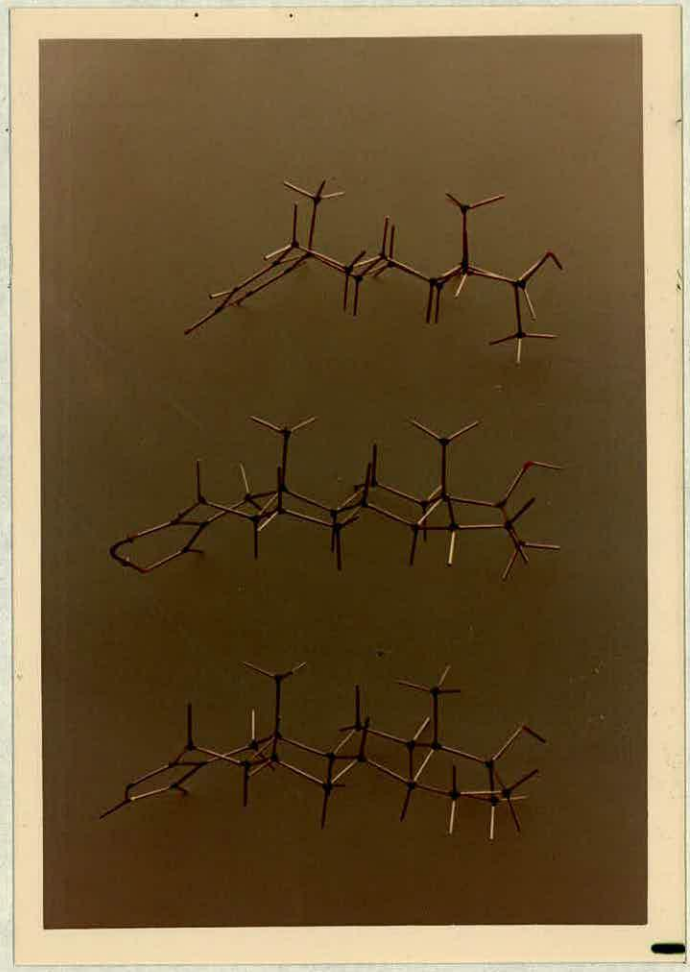


Figure 2. Dreiding models of methandrostenolone, oxymetholone and 17β-hydroxy-17-methyl-5α-androstane-(3,2-c)-pyrazole.

be a potent oral anabolic agent with minimal androgenic activity and is marketed as Adroyd (Parke-Davis) and Anandrol (Syntex).

At first sight the structure of oxymetholone[5] appears to be quite different from that of methandrostenolone [4]. However, a Dreiding model of the former compound shows that the hydrogen bonding between the hydroxymethylene hydroxyl group and the C-3 carbonyl group leads to the formation of a flat six-membered ring fused to the A-ring. The model also shows that this additional "ring" is bent towards the α-side of the molecule, which suggests the possibility that the ability of oxymetholone and methandrostenolone to produce anabolic activity may be related to their geometry at the A-ring extremity of each molecule [see figure 2].

This hypothesis is in accordance with the concept, outlined earlier (page 5), that different structural features of the molecule may be responsible for different requirements for hormonal activity. Thus, both methandrostenolone and oxymetholone possess the 17β-hydroxy-17-methyl function, which confers oral stability, and both contain a flat ring at the other end of the molecule, bent towards the α-side. This feature might then be responsible for the effect on the protein synthesising system. In this case the difference between the lengths of the molecules of methandrostenolone and oxymetholone, the latter having an extra ring, between the two active sites mentioned above could be expected to be unimportant.[5d] Oxymetholone is similar, with respect to the additional flat "ring", to the group of anabolic steroids in

which a heterocyclic ring is fused to C-2 and C-3 [figure 2].

The metabolic pathways of the body act as defence mechanisms to facilitate the removal of potentially dangerous foreign compounds.[21a] The major metabolic transformations, such as oxidation, reduction and hydrolysis result in the introduction of new functional groups and may lead to activation, as typified by the formation of 19-nortestosterone from its esters, or deactivation by the introduction of centres for further catabolic reactions. The other major mode of detoxication is conjugation, in which functional groups are masked by reaction with endogenous substrates, which are chiefly sulphuric and glucuronic acids in steroid metabolism. The polarity of the molecule is thus increased, it is less lipid-soluble and more easily excreted. The metabolism of steroids and other foreign compounds is effected mainly by enzymes found in the liver and the metabolites are excreted in the urine.

The metabolism of the natural androgens has been well studied[22,23] but that of the majority of synthetic anabolic steroids has yet to be established.[5e] Recently, however, several groups of workers have been engaged in the examination of the metabolism of a variety of these compounds.[24-27] Probably the most important factor which has contributed to the rise in the number of such investigations is the rapidly increasing use of mass spectrometry.

Knowledge of the metabolism of anabolic steroids is of interest for a variety of reasons. The question of whether metabolism may lead to compounds with activity similar to that

of the parent drug or with completely different activity is of considerable importance.[5e] Identification of the metabolites may yield information regarding drug toxicity[21b,28] and in some situations may be the only means of establishing drug administration.

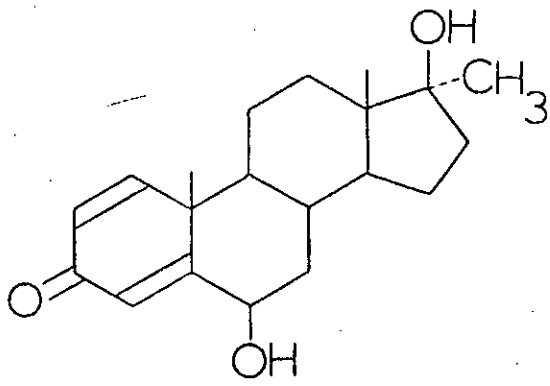
Foreign compounds such as drugs usually give rise to a considerable number of different metabolites because of the variety of biotransformation and conjugation pathways which may operate. The metabolic pattern and the toxicity of a drug may be altered by genetic, physiological or environmental factors which affect the rate of reactions and thus the relative importance of each metabolite.[21b] Of interest in the clinical situation are the physiological effects of age, sex, nutritional state, pregnancy and disease and the environmental effects of stress due to adverse conditions, exposure to ionising radiation and the ingestion of other foreign compounds.

In order to draw valid conclusions from studies of the metabolism of anabolic steroids under such conditions a knowledge of the metabolism of the compounds in the normal healthy individual is essential. These investigations have been limited by the sensitivity of the techniques available for detection and identification of metabolites since the administration of large doses of steroids to normal subjects not undergoing treatment is unjustifiable. Methods employing small doses of radioactively labelled steroids have been used successfully[29-31] but suitably labelled compounds are generally difficult to obtain and the danger from exposure to radioactivity would, under most circumstances, preclude the study

of the metabolism of children and pregnant women.[32]

Adhikary and Harkness[33] have developed a method by which microgram quantities of steroids can be detected as the parent steranes. The technique, known as carbon skeleton chromatography, involves the high temperature catalytic reduction of the steroid to its hydrocarbon skeleton and analysis of the trapped reduction products by gas-liquid chromatography.[34] The metabolism of synthetic steroids which possess skeletons not found among the naturally-occurring steroids, such as 19-nortestosterones and 17-alkylandrostanes, is particularly suited to examination by this method.[35,36] Crude metabolic extracts are partially separated by conventional chromatography on thin layers or paper. A small sample from each fraction is examined by carbon skeleton chromatography for metabolites bearing the skeleton of the drug. Those fractions which contain metabolites can thus be isolated, further purified and examined by other means in order to establish their identity.

Catalytic reduction of steroids at 170-200°C is a harsh procedure and only 5-10% of tri-oxygenated androstanes are recovered as the steranes, compared to 50% recovery from mono-oxyandrostanes.[34] The reliability of the method has, however, been extensively investigated by the reduction of known steroids and of the metabolites of drugs which have also been examined by conventional methods.[32,34,36,37] The success of the technique as applied to the metabolism of steroid drugs with skeletons other than those of the natural products depends on the retention of this unnatural skeletal "label" during



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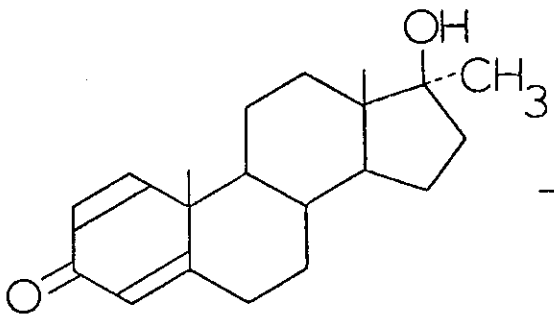

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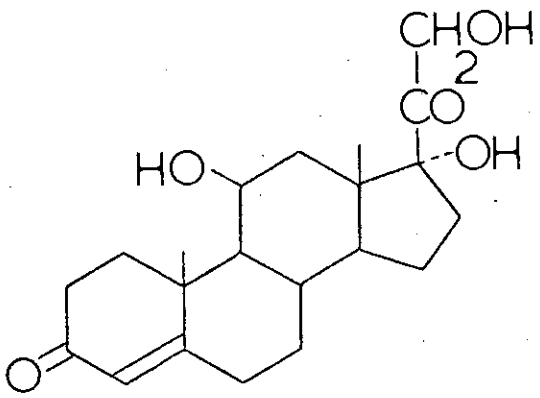
metabolism. It has been repeatedly shown that the carbon skeletons of the steroids remain largely intact during metabolism;[5e,31,38-40] consequently the method has been used for the study of the metabolism of normal therapeutic doses of anabolic steroids.[32,37]

Carbon skeleton chromatography is a useful technique for the location of microgram quantities of metabolites of anabolic steroid drugs in biological extracts. However standard compounds which have been synthesised by unambiguous routes are essential for the complete identification of any metabolites, particularly those isolated in very small amounts.[41] The work described here includes the synthesis of compounds as standards for the metabolites of methandrostenolone and oxymetholone.

The metabolism of methandrostenolone was first investigated by Rongone and Segaloff[42] in 1963 following the successful isolation of A-ring-reduced metabolites from 17α-methyltestosterone[43] and 17a-oxa-D-homoandrosta-1,4-dien-3-one (Δ^1-testololactone).[44] After administration of methandrostenolone[4] two metabolites were isolated, both of which however possessed the intact $\Delta^{1,4}$-dien-3-one system. The major metabolite was positively identified as 6β-hydroxymethandrostenolone[9] by comparison with the authentic material. The second metabolite was not structurally identified although the data indicated that it was an isomer of methandrostenolone rather than a hydroxylated product.

Sandor and Lanthier[45] studied the metabolism of a small dose, comparable to the physiological level, of 17α-



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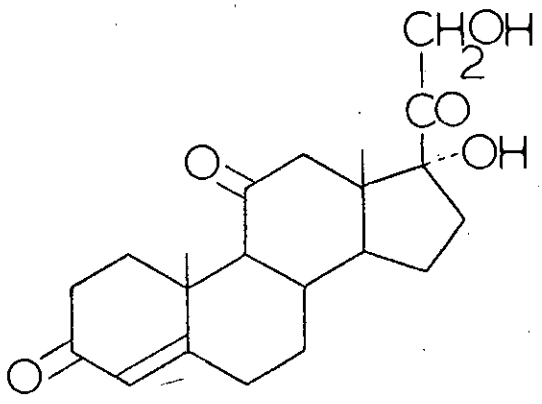

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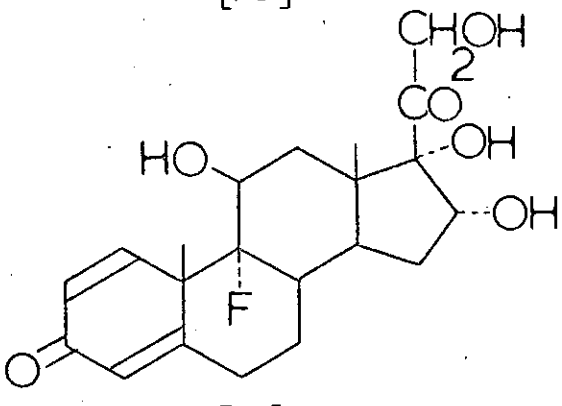

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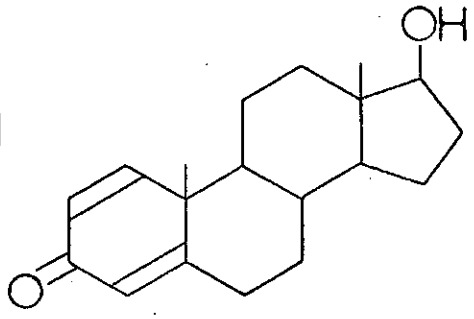

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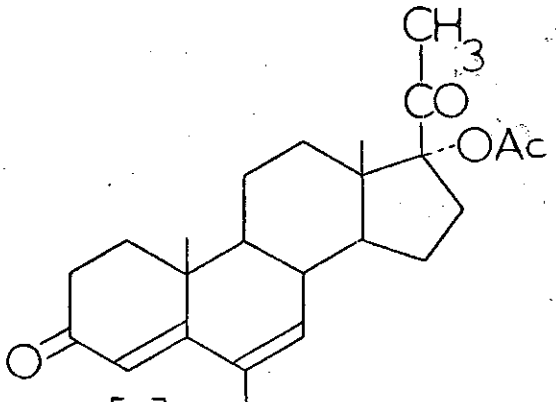

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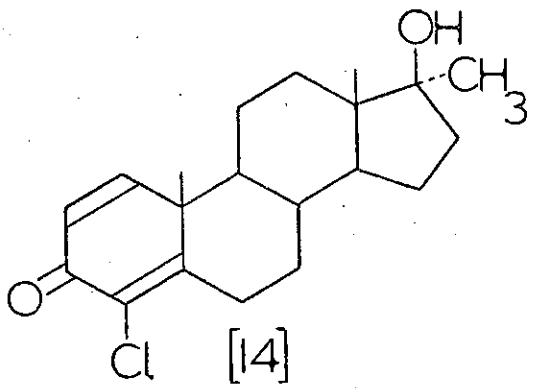

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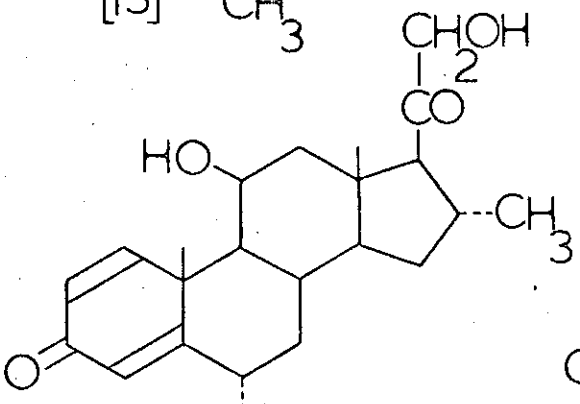

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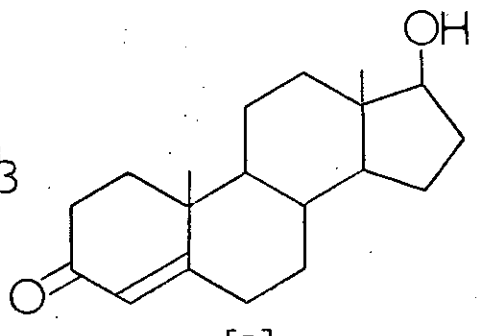
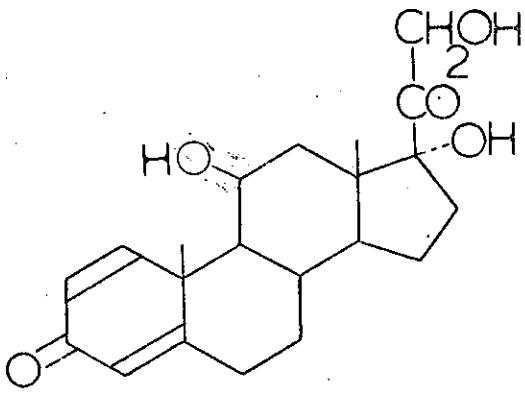


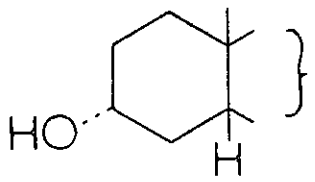
[8]

^{14}C-methandrostenolone in the dog. They found that the drug had a long biological half life (132 minutes) in comparison to cortisol (52 minutes)[46] and attributed this to the resistance of the A-ring and C-17 substituents to enzymatic transformation. Most of the metabolic products were unconjugated; Adhikary and Harkness[37] likewise found no conjugated metabolites of methandrostenolone in the human. The majority of the radioactivity (85.4%) administered to the dog was excreted in urinary metabolites within 72 hours of injection although only 38% was recovered in the crude chloroform extracts. The remaining material was extracted with solvents of higher polarity. Since the material in these extracts was similar to that extracted with chloroform it was suggested that the phenomenon was due only to partition of the highly polar metabolites between chloroform and water. Low recoveries of metabolites of methandrostenolone[37] and methyltestosterone[43] by chloroform extraction have been reported elsewhere. Two metabolites of methandrostenolone were isolated[45] from the dog and it was postulated that these were C-6 oxygenated products.

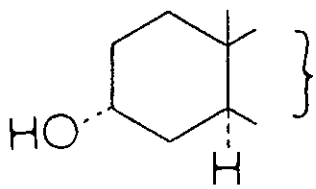
The 6β-hydroxylation of steroids containing the Δ^4-en-3-one and $\Delta^{1,4}$-dien-3-one systems is now an established biochemical pathway. Cortisol[10],[47,48] cortisone[11][49] and 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione (triamcinolone)[12][50] have been shown to be metabolised to their 6β-hydroxy derivatives by a variety of tissues.[49,51,52] More recently the biochemical 6β-hydroxylations of 17β-hydroxyandrosta-1,4-dien-3-one[7],[26,53] 17α-hydroxy-6-methylpregna-4,



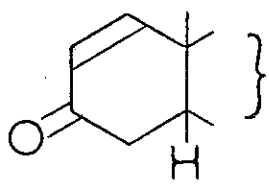
[16]



[17]



[18]

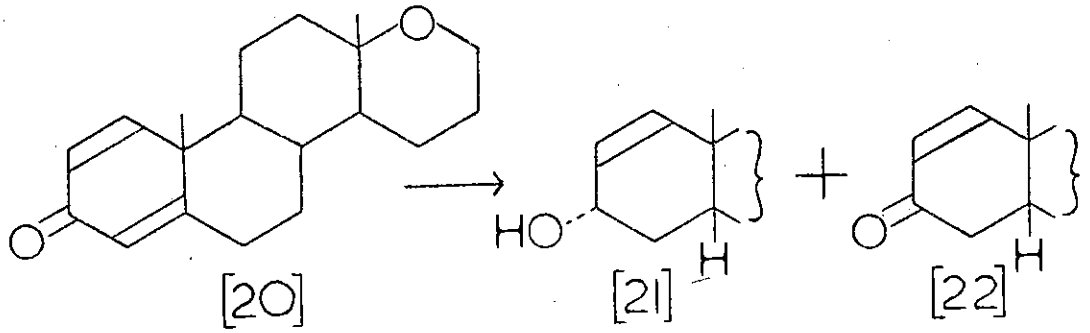


[19]

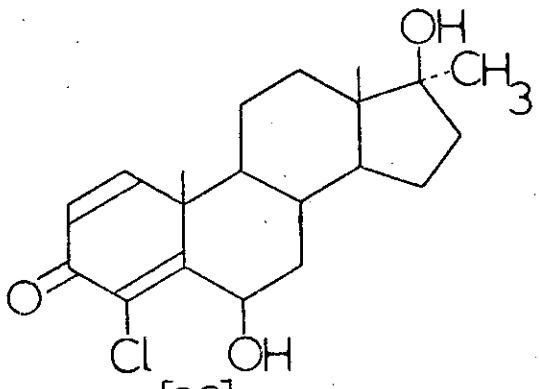
6-diene-3,20-dione 17-acetate [13],[31] 4-chloro-17β-hydroxy-17-methylandrosta-1,4-dien-3-one [14],[24,54] 11β,21-dihydroxy-6α-fluoro-16α-methylpregna-1,4-diene-3,20-dione [15][55] and testosterone [8][56] have also been reported.

The metabolism of compounds containing the 1,4-dien-3-one system was first investigated[57-60] following the discovery[61] that the antiinflammatory drugs prednisone and prednisolone were more active than their 1,2-dihydro analogues, cortisone and cortisol. Failure to demonstrate any ring-A reduction products from prednisone and prednisolone, in contrast to the metabolism[62,63] of cortisone and cortisol, led to the suggestion[57,58] that the increased biological activity was due to the resistance of the 1,4-dien-3-one system to enzymatic attack. Later, however, Caspi and Pechet[64] isolated metabolic products of prednisolone [16] containing the 3α-hydroxy-4,5β-dihydro [17], 3α-hydroxy-4,5α-dihydro [18] and 1-en-3-one-4,5β-dihydro [19] A-rings. Various studies, *in vivo*[65] and *in vitro*,[66-68] have demonstrated the inhibition of A-ring reduction by 1,2-dehydrogenation in the corticosteroids. The principle metabolite of one of the most active of these compounds, triamcinolone [12], is the unconjugated 6β-hydroxy derivative.[50]

Metabolic reduction of the 1,4-dien-3-one system of Δ^1-testololactone [20] has been shown to occur to give the Δ^1-ene-3α-hydroxy-4,5β-dihydro [21][44] and Δ^1-ene-3-keto-4,5β-dihydro [22][69] derivatives. The A-ring remained intact in 37-51% of the material isolated after the administration of 17β-hydroxyandrosta-1,4-dien-3-one,[53] its 17-cyclopentenyl



[20] [21] [22]



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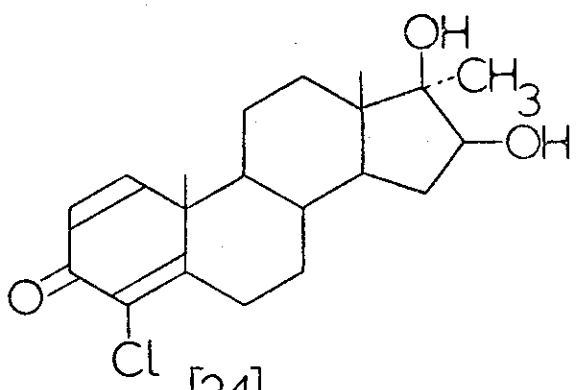
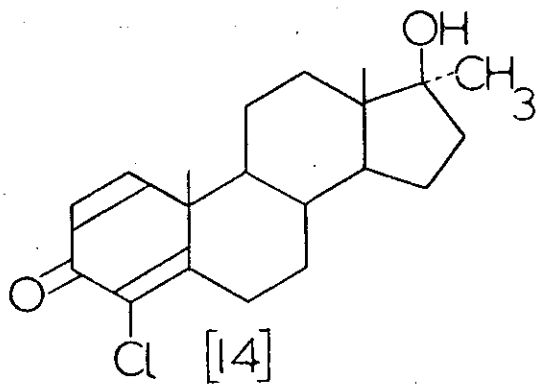
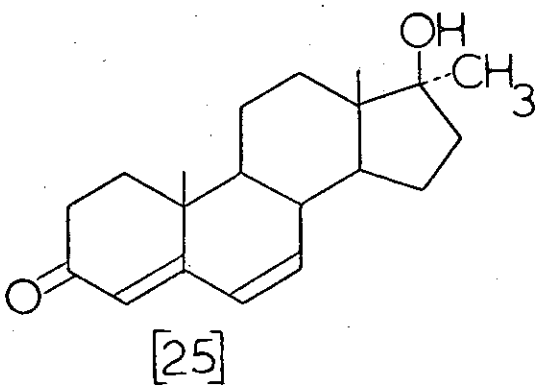


[24]



[14]



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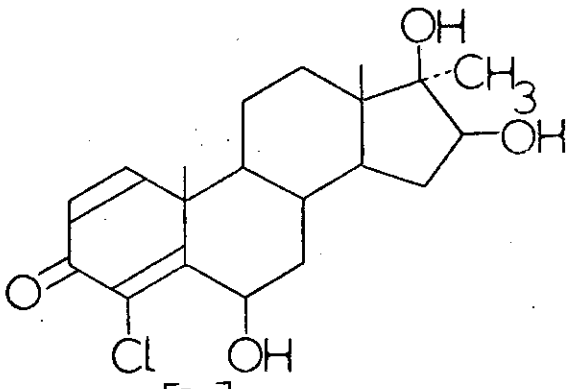
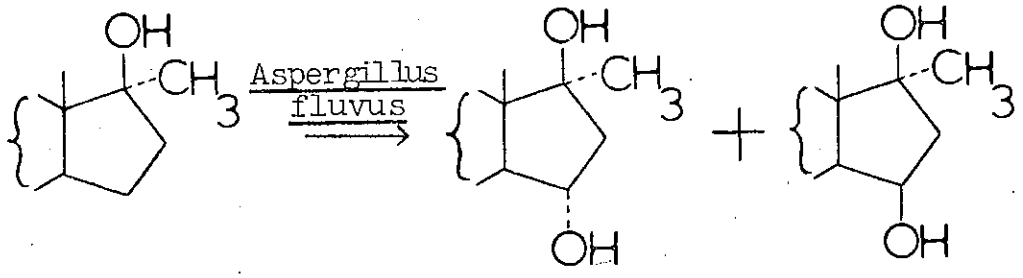


[26]

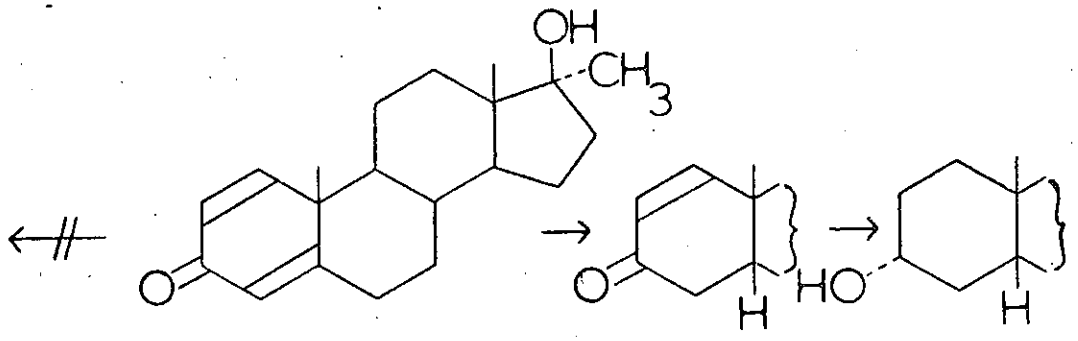
ether and androsta-1,4-diene-3,17-dione to normal men.[26] Less than 5% by weight of the metabolites contained the fully A-ring-reduced 3α-hydroxy-4,5β-dihydro structure. The remainder of the products were 5β-androst-1-enes with 3α-hydroxy or 3-keto substituents.

Schubert and Wehrberger[24] have examined the metabolism of the 4-chloro analogue of methandrostenolone, 4-chloro-17β-hydroxy-17-methylandrosta-1,4-dien-3-one[14], and isolated ca. 20% of the dose as crude metabolic fractions from which they obtained twenty-one metabolites accounting for 1.5% of the administered drug. The major metabolite was reported to be the sulphate conjugate of the unchanged drug which, with the 6β-hydroxy and 16β-hydroxy derivatives[23,24], accounted for 86% by weight of the metabolites. Of the eighteen other metabolites only one, 17β-hydroxy-17-methyl-androsta-4,6-dien-3-one[25], was positively identified. All but one of the seventeen unidentified metabolites were thought to contain the intact 1,4-dien-3-one A-ring of the drug. Schubert and Schumann[54,70] have since identified the 6β,16β-dihydroxy derivative[26] as a metabolite of this steroid.

The microbial metabolism of testosterone, 17α-methyl-testosterone, methandrostenolone, 4-chloro-17α-methyltesto-sterone and 4-chloro-17β-hydroxy-17-methylandrosta-1,4-dien-3-one has recently been reported by Schubert et al.[71] The 17α-methyl steroids[27] were hydroxylated by Aspergillus fluvus at C-15, in contrast to the B-ring hydroxylation of testosterone. The ratio of 15α- to 15β-hydroxylation[28,29] appeared to depend upon the nature of the A-ring substituents.



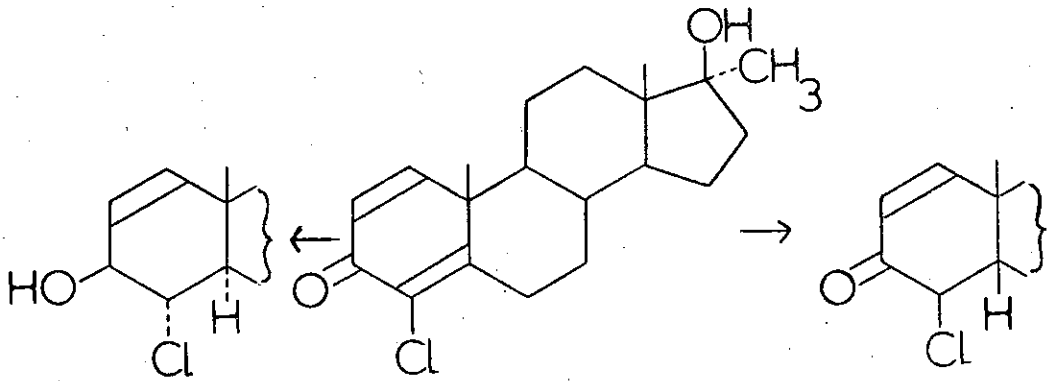
[27] [28] [29]



[4] [30] [31]

Rhodotorula glutinis

Clostridium paraputrificum



[33] [14] [32]

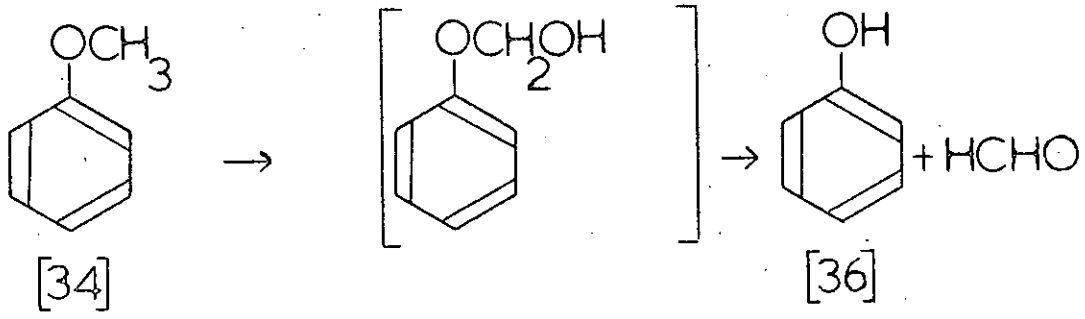
Methandrostenolone[4] was metabolised by Clostridium paraputrificum to the Δ^1-ene-3-keto-4,5β-dihydro compound [30] which was further transformed to 17α-methyl-5β-androstane-3α,17-diol[31]. The Δ^1-double bond of 4-chloro-17β-hydroxy-17-methylandrosta-1,4-dien-3-one[14] was not reduced by this organism[32]. Methandrostenolone was not metabolised by Rhodoturula glutinis, although its 4-chloro analogue[14] was transformed to the Δ^1-ene-3β-hydroxy-4α-chloro-5α-steroid[33] and the other compounds were reduced to 3-hydroxy-5α-androstanes.

The question of whether the 1,4-dien-3-one system is aromatised to a significant extent during metabolism is important because of the changed biological activity of the resulting metabolite[5e] and because an oestrogen would not be detected as a metabolite of a 17α-methylandrostane by carbon skeleton chromatography. Aromatisation of 17β-hydroxy-androsta-1,4-dien-3-one,[72] androsta-1,4-diene-3,17-dione[73] and methandrostenolone[5e] by placental enzymes has been demonstrated but no oestrogens were detected following the incubation of methandrostenolone with rat liver slices.[74] In a study[70] of tritium-labelled 4-chloro-17β-hydroxy-17-methylandrosta-1,4-dien-3-one in normal non-pregnant women less than 1% of the radioactivity was detected in 17α-methyl-oestra-1,3,5(10)-triene-3,16α,17-triol, consistent with the known low level of aromatisation of neutral steroids.[73]

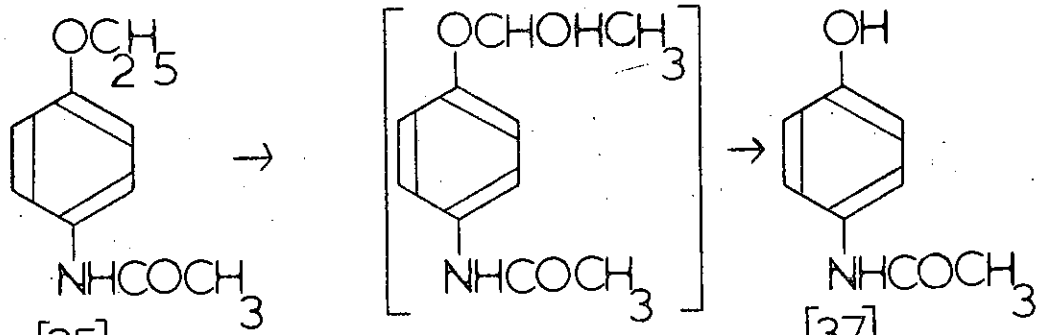
After the administration of 4g of methandrostenolone to a woman with advanced adenocarcinoma of the lung Rongone and Segaloff recovered 7.25% of the dose as two crystalline

metabolites.[42] The major metabolite, 6β-hydroxymethandrostenolone, accounted for 80% of the extracted material. During their study of the reliability of carbon skeleton chromatography Adhikary and Harkness also identified two metabolites of methandrostenolone from normal men who were given the therapeutic dose of 5mg of the drug.[37] However, in this case 6β-hydroxymethandrostenolone was present as only a third of the extract. The predominance of the 6β-hydroxylation pathway in the first investigation may be due to a variety of factors stemming from the condition of the subject. Experiments with rats have shown that microsomal enzymes are stimulated by pretreatment with steroid hormones to a degree related to the anabolic activity of the compound.[75] A large dose of an anabolic steroid might thus stimulate its own hydroxylation. It is also well-known that many drugs stimulate or inhibit microsomal metabolism of other drugs,[76-79] an effect which is important in multiple prescription,[21c] and that hydroxylation is dependent upon the condition of the person to whom the drug is administered.[28]

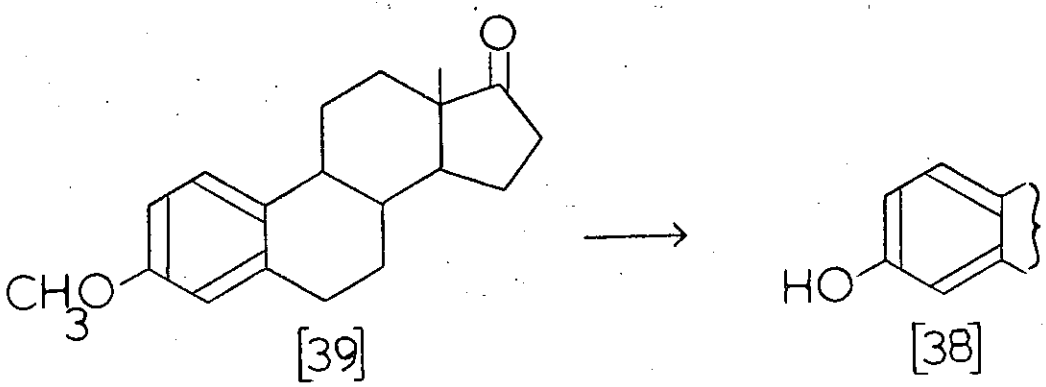
Adhikary and Harkness used carbon skeleton chromatography to examine the metabolites of the therapeutic dose of 10mg of the anabolic steroid drug oxymetholone in normal man.[32,37] The metabolism of this compound has not been reported elsewhere. The retention times of the reduction products from oxymetholone were identical to those from methandrostenolone,[36] indicating that the additional C-C bond to the 2-position in oxymetholone is labile under the hydrogenation conditions employed. Thus the presence or absence



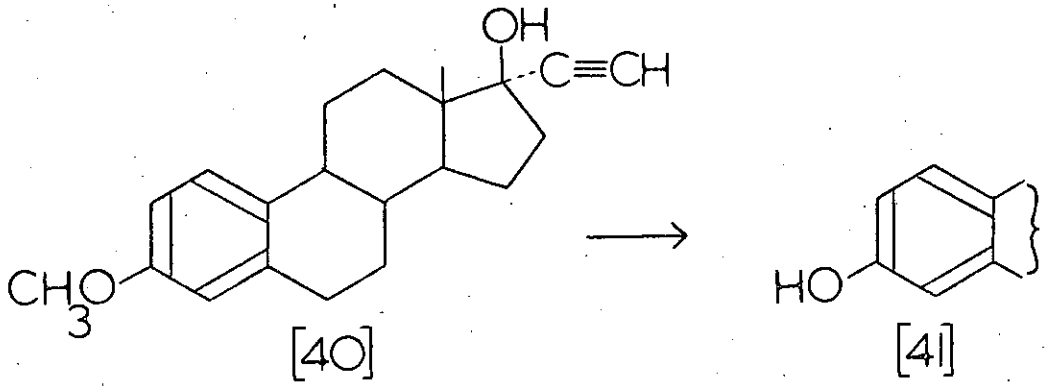
[34] [36]



[35] [37]



[39] [38]



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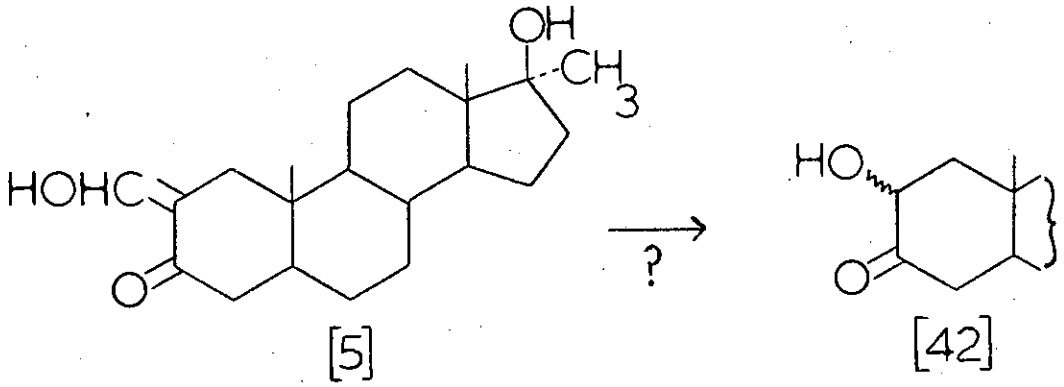
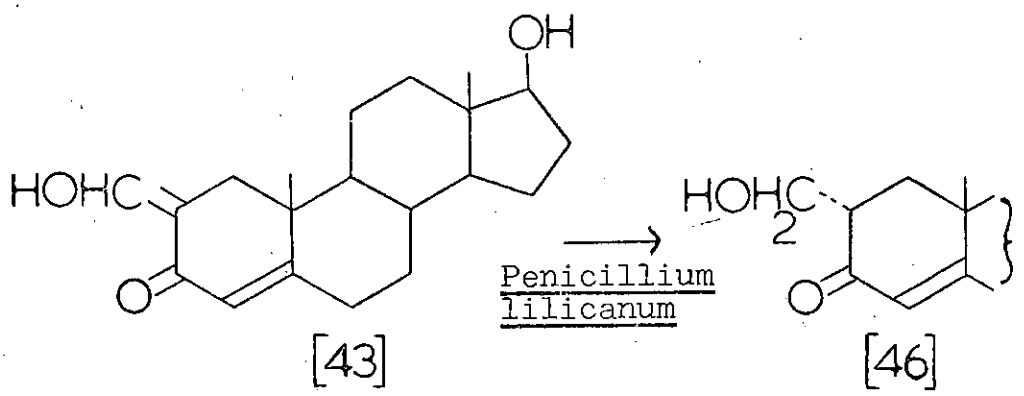


[5] [42]

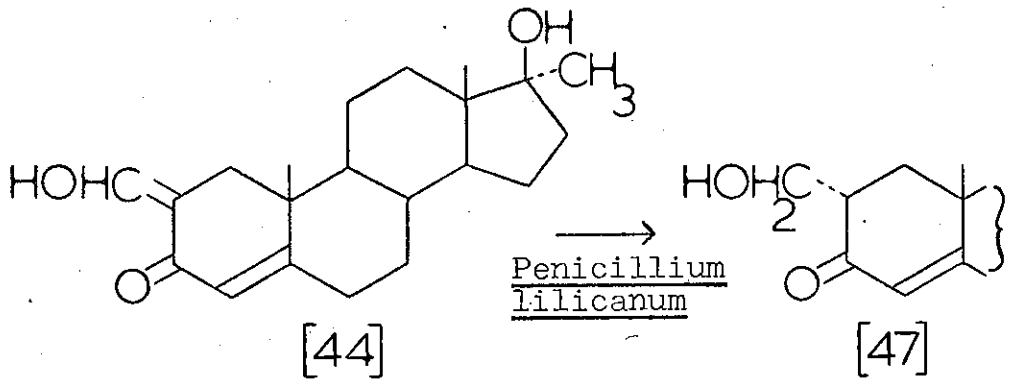
of the additional carbon atom in the two metabolites could not be inferred.

Aromatic methyl[34] and ethyl ethers[35] are dealkylated to the corresponding phenol[36,37] by microsomal enzyme systems[21] and oestrone[38] has been assumed to arise from 3-methoxyoestrone[39] by this pathway.[80] Rat liver microsomes have been shown[81] to O-demethylate 17α-ethynyl-17-hydroxy-3-methoxyoestra-1,3,5(10)-triene(mestranol)[40] to ethynyloestradiol[41] and the slow release of the active phenol is thought to be the reason for the reported enhancement of the activity of ethynyloestradiol by formation of the 3-ether. Adhikary[32] suggested that the C-2 substituent of oxymetholone[5] might be removed in this manner to yield 2-hydroxy metabolites[42]. However a study of the metabolism of 1-^{14}C-methyl-17β-hydroxy-5α-androst-1-en-3-one in the rat showed that no biological dealkylation of this compound occurred.[82]

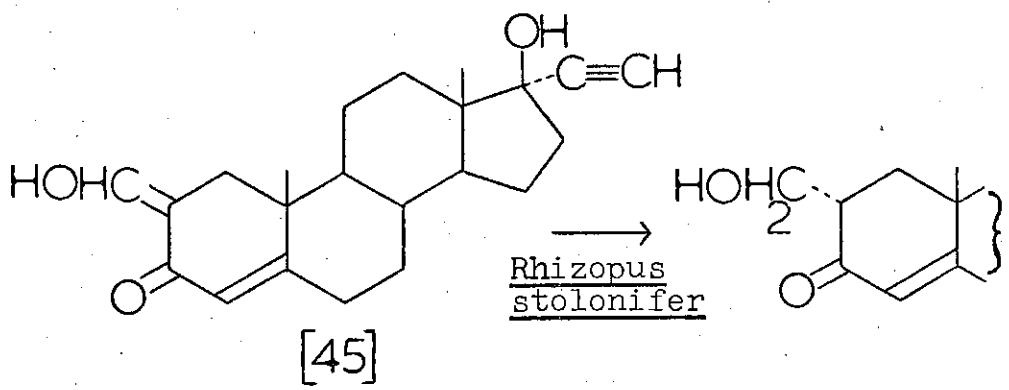
The microbial transformations of 2-hydroxymethylene-17β-hydroxyandrost-4-en-3-one[43], its 17α-methyl[44] and 17α-ethynyl[45] analogues with a variety of organisms were investigated by Nielson et al.[83] Penicillium lilicanum reduced the 17β-hydroxy[43] and 17β-hydroxy-17-methyl[44] compounds to the 2α-hydroxymethyl derivatives[46,47]. The 17β-hydroxy-17-ethynyl compound[45] was similarly reduced by Rhizopus stolonifer.[84] Neither of these organisms introduced additional hydroxyl functions. 6β-Hydroxyl and 16α-hydroxyl groups were introduced by other organisms, together with reduction of the 2-hydroxymethylene function.


OH
HOHC
O
Penicillium lilicanum
HOHC
2
O

[43] [46]


OH
CH3
HOHC
O
Penicillium lilicanum
HOHC
2
O

[44] [47]


OH
C≡CH
HOHC
O
Rhizopus stolonifer
HOHC
2
O

[45]

Goldman[85] has investigated the effects of oxymetholone as an inhibitor of 3β-hydroxysteroid dehydrogenase and Δ^{5-4}-3-ketosteroid isomerase in the rat. He found that NADH was formed from NAD and the steroid at a rate which suggested that oxymetholone was itself dehydrogenated in the system.

In view of the successful isolation of two major metabolites from both methandrostenolone[32,37,42,45] and oxymetholone[32,37] and the previous characterisation[42] of only one of these products it was deemed appropriate to commence a systematic study into the nature of the unknown metabolites. The limited information concerning the identities of the three compounds has now been summarised and the results of the investigations are described in the following sections.

2. The Extraction, Location and Purification of the Metabolites of Methandrostenolone and Oxymetholone

Adhikary[32] studied the use of high temperature catalytic reduction of steroids for the identification of the metabolites of steroid drugs containing carbon skeletons not found among the natural products. He confirmed the previous reports[42,45] of two major metabolites of methandrostenolone and demonstrated that the hitherto unstudied anabolic agent, oxymetholone, was converted to two major metabolites in normal men.

The production, extraction and isolation of the metabolites of methandrostenolone and oxymetholone were carried out at the Department of Paediatric Biochemistry, Royal Hospital for Sick Children, Edinburgh by Dr. R.A. Harkness, Dr. P.M. Adhikary, Mr. A.M. Torrance and Mr. B. Kilshaw.

Each healthy male volunteer received the normal therapeutic dose of one 5mg tablet of methandrostenolone or one 10mg tablet of oxymetholone. An examination, described later (section 3.7), of the rate of excretion of the less polar, previously unidentified metabolite of methandrostenolone showed that excretion reached a maximum and fell to a constant low value within the first 24 hours following drug administration. Consequently urine was not normally collected beyond this period after administration of either drug.

The two metabolites of methandrostenolone are not conjugated with glucuronic or sulphuric acids and are recovered from urine by chloroform extraction (free fraction). No metabolites of oxymetholone were however detected in the

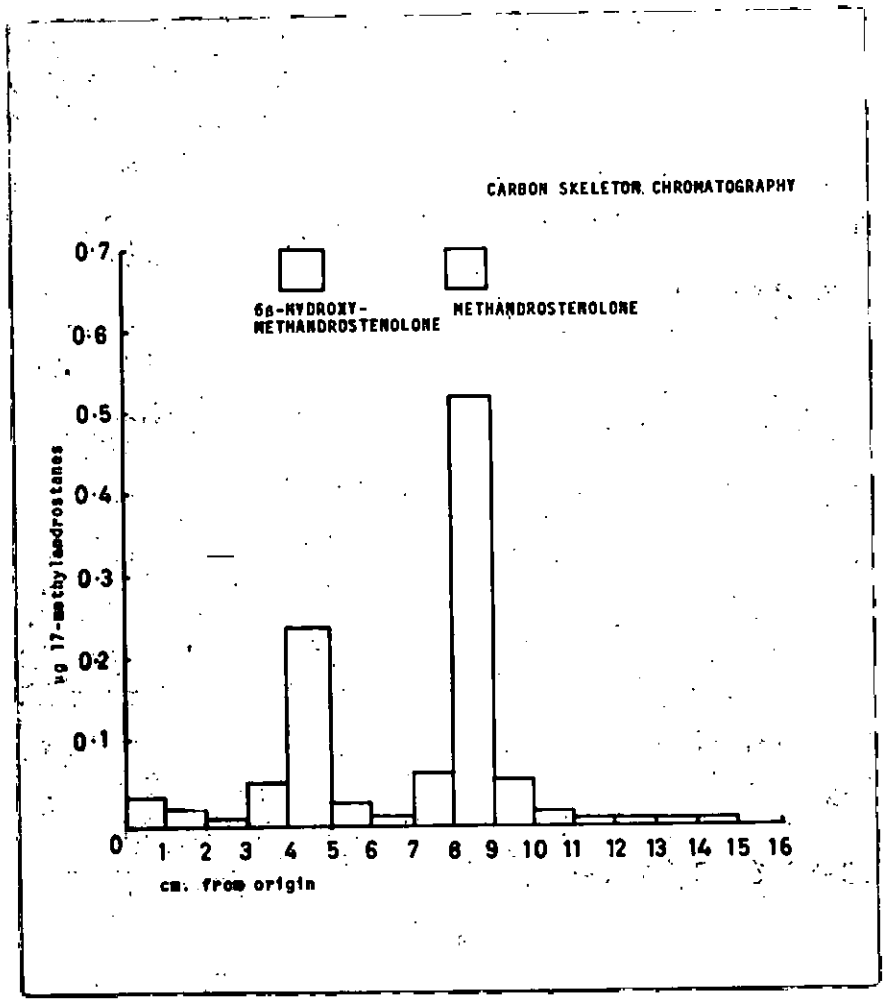


Figure 3. The freely extractable urinary metabolites of methandrostenolone separated by thin layer chromatography and detected by carbon skeleton chromatography. The chromatographic behaviour of the named reference compounds is shown by the top squares.

free fraction. Both are excreted as glucuronide conjugates and are recovered by hydrolysis of the urine with β-glucuronidase prior to chloroform extraction (glucuronide fraction).

The chloroform extracts of the free and glucuronide fractions were washed with dilute sodium hydroxide solution and then with distilled water until neutral, dried over anhydrous sodium sulphate and evaporated to dryness. The residues were dissolved in ethanol and chromatographed on thin layers of silica gel G in the solvent system, chloroform-methanol (9:1 or 19:1). The plates were divided into 1cm bands and the material eluted from each band. These eluates were halved, evaporated to dryness and one set of residues dissolved in ethanol (0.1ml). 10μl of this solution from each fraction was used for high temperature catalytic reduction at 180-190°C over a 1-3% w/w platinum catalyst. The reduction products were analysed by gas chromatography on 1% SE-30 or 1% NGA columns. A hydrocarbon standard consisting of 17-methyl-5α- and 17-methyl-5β-androstanes was derived from the drug by the same method. Those bands which contained material with the same hydrocarbon skeleton as the drug were thus identified and further purified by thin layer and paper chromatography. Carbon skeleton chromatography was repeated at each stage of the purification to confirm the presence of compounds with the skeleton of the drug.

The more polar of the two metabolites of methandrostenolone [see figure 3] behaved similarly to 6β-hydroxymethandrostenolone, the major metabolite identified by Rongone and

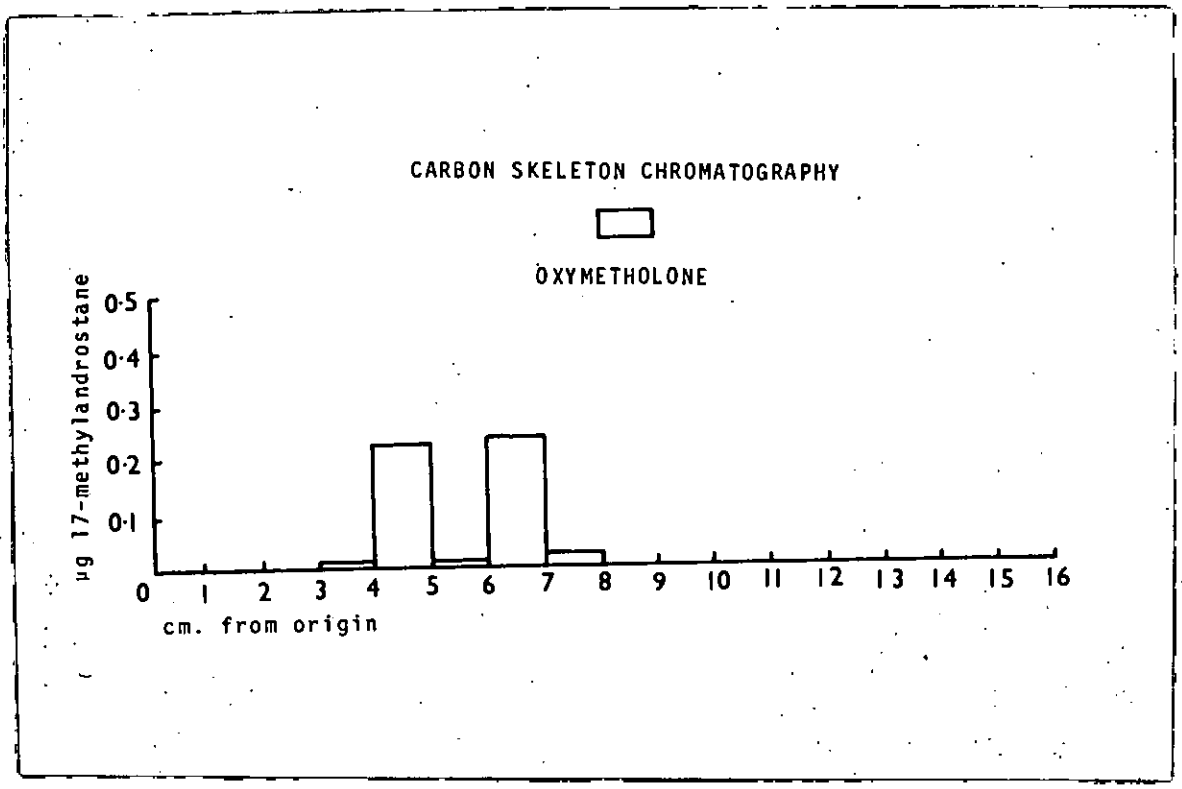


Figure 4. The conjugated metabolites of oxymetholone separated by thin layer chromatography and detected by carbon skeleton chromatography. The chromatographic behaviour of oxymetholone is shown by the top rectangle.

Segaloff;[42] it was not further studied. The second metabolite, M, appeared identical to methandrostenolone when compared by thin layer and paper chromatography. However, gas-liquid chromatography on SE-30 and QF-1 columns showed that M had a slightly shorter retention time than methandrostenolone.[32] Approximately 5% of the dose was isolated as 6β-hydroxymethandrostenolone and M, in the ratio 1:2, assuming that the response of both compounds to carbon skeleton chromatography was the same.

About 5% of the dose of oxymetholone was isolated as two metabolites [see figure 4], both more polar than the drug and in the ratio 1:1 assuming equal response to carbon skeleton chromatography.

3.1 The Synthesis of 17-Epimethandrostenolone

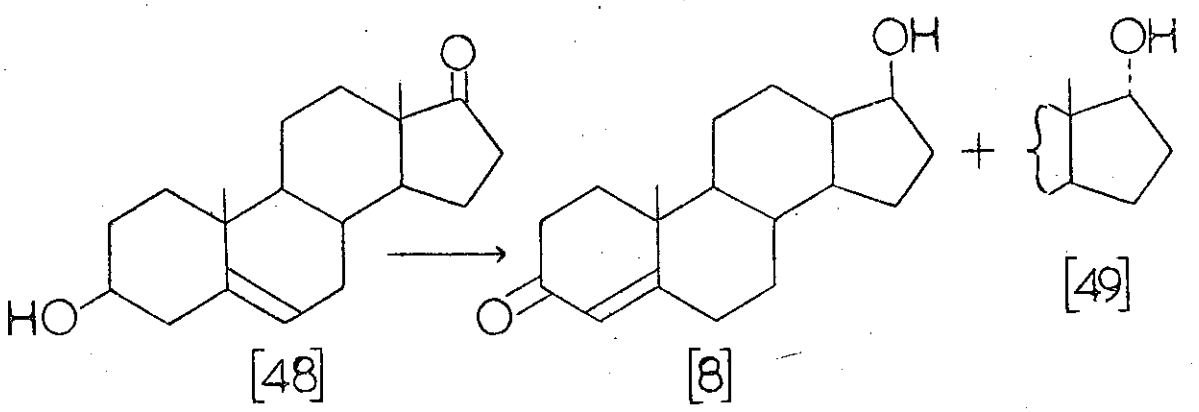
3.1.i. Rongone and Segaloff[42] purified sufficient of their unidentified metabolite of methandrostenolone for analysis by ultraviolet and infrared spectroscopy, both of which indicated that the compound possessed the 1,4-dien-3-one system. Elemental analysis showed that its composition was closer to that of methandrostenolone than an oxygenated product but the melting point (222-224°C) demonstrated that it was not methandrostenolone (m.p. 164-166°C). In view of this evidence it was suggested that the metabolite might be an isomer of methandrostenolone.

Adhikary[32] recovered about 3.75% (ca. 160µg) of administered methandrostenolone as a metabolite, M, with similar polarity to the drug in thin layer and paper chromatographic systems. M was only distinguishable from methandrostenolone by gas-liquid chromatography [table 1].

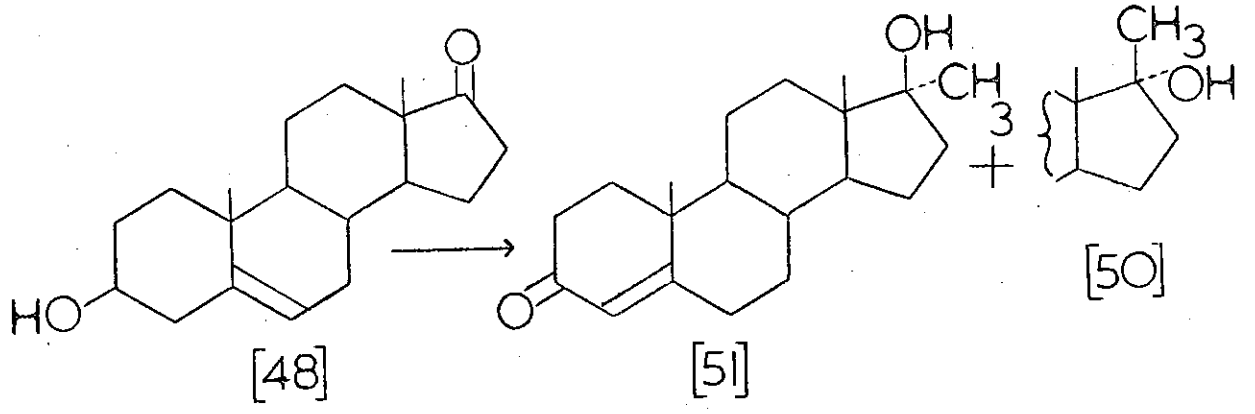
Column	SE-30	QF-1
Compound	Retention time (mins.)	
Methandrostenolone	23.0	25.3
Metabolite M	22.0	23.9

Table 1. The retention times of methandrostenolone and its less polar metabolite M on SE-30 and QF-1 columns

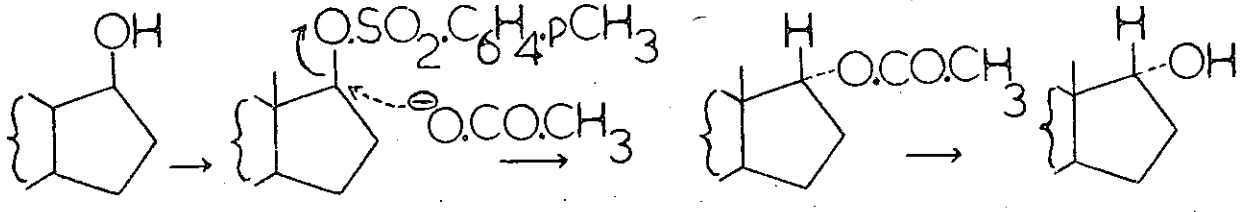
From the preceding evidence it was postulated that M might be the 17-epimer of methandrostenolone, 17-epimethandrostenolone. This compound could be expected to exhibit similar ultraviolet, infrared and chromatographic behaviour



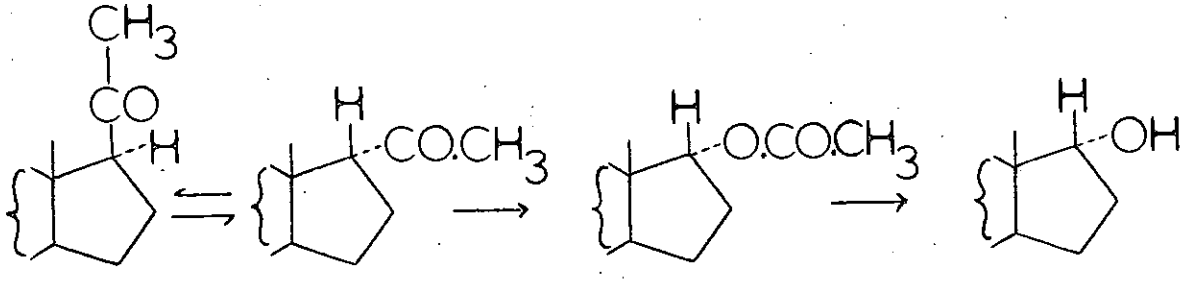
[48] [8] [49]



[48] [51] [50]



[52] [53] [54]



[57] [56] [55]

to methandrostenolone since epimerisation of the C-17 substituents should have very little effect on the shape of the molecule. The synthesis of 17-epimethandrostenolone has not been previously reported.

3.1.ii. Previous syntheses of 17-epitestosterone and 17-methylepitestosterone

During the synthesis of testosterone[8] by reduction of the 17-carbonyl group of dehydroepiandrosterone[48] Ruzicka and Kägi,[86] in 1936, obtained a small amount of 17-epitestosterone [49]. Similarly, in 1939 Miescher and Klarer[87] isolated 17-methylepitestosterone[50] in very low yield during the synthesis of methyltestosterone [51] by the Grignard reaction of methylmagnesium iodide with dehydroepiandrosterone [48]. These compounds were found to possess very low androgenicity in comparison to their highly active C-17 epimers. There was no interest in 17α-hydroxyandrostane derivatives until Sondheimer et al.[88] devised methods for the preparation of 17-epitestosterone and 17-methylepitestosterone in yields large enough to permit their testing as anabolic agents. No such activity was detected and so, although many steroid drugs possess the 17β-hydroxy-17-methyl function few C-17 epimers with the 17α-hydroxyl group have been reported. Ananchenko et al.[89] found that a series of D-homo-17aβ-alkyl-17a-alcohols were less androgenic and less anabolic than their 17aβ-hydroxy epimers.

No methods had been devised for the preparation of 17α-hydroxyandrostanes in reasonable yields prior to those of Sondheimer et al.[88] Because the C-17 carbonyl group is

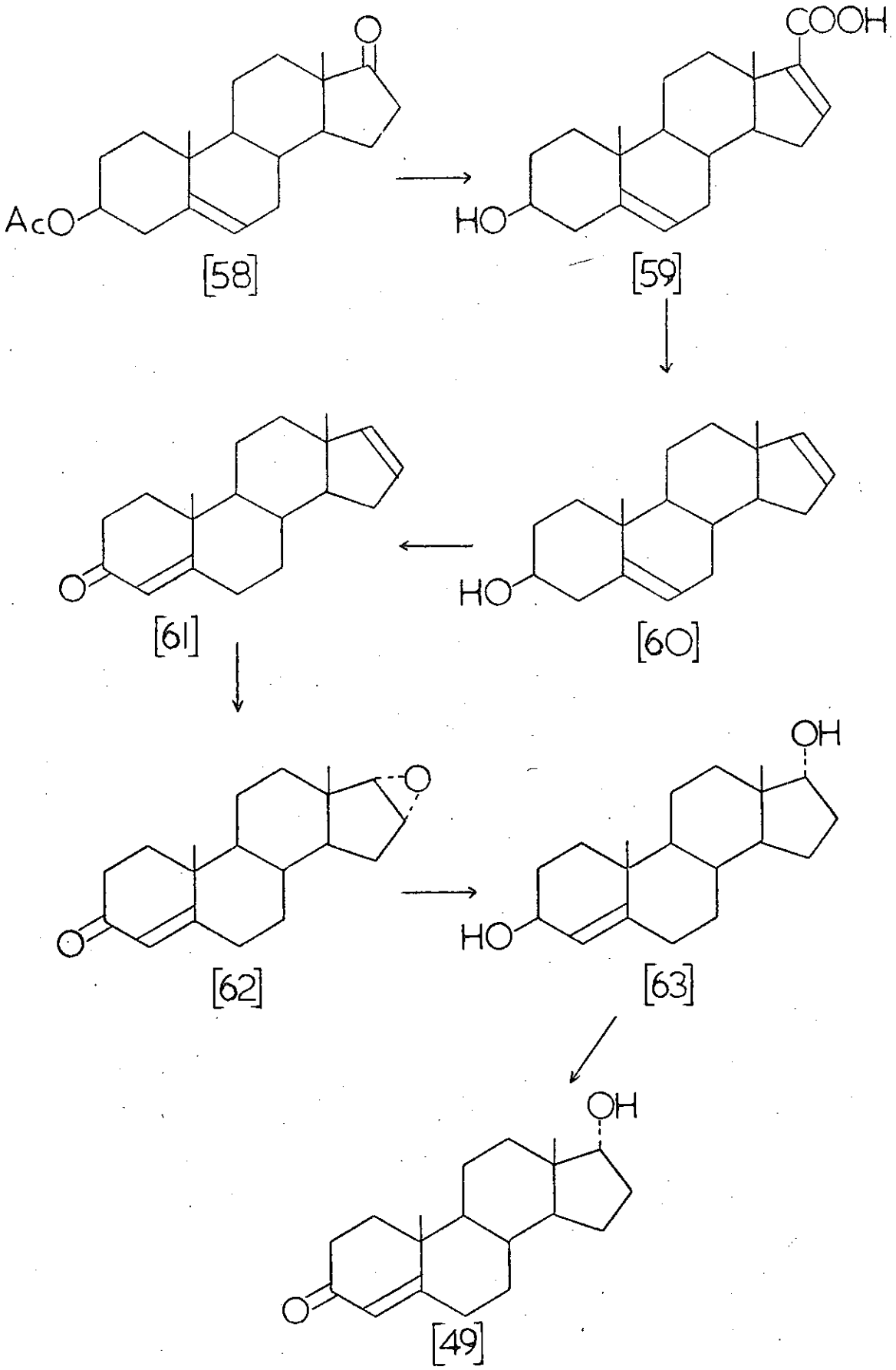


Diagram 1. Previous synthesis of 17-epitestosterone

subject to predominantly α-face attack the methods of Ruzicka and Kägi[86] and Miescher and Klarer[87] were of no preparative value. Conversion of a 17β-alcohol[52] to the toluene-p-sulphonate derivative[53] and subsequent acetolysis and saponification led to the 17α-ol[54] in ca. 5% yield.[90,91] The preparation of 17α-hydroxy compounds[55] by perbenzoic acid oxidation of 17-isopregnane derivatives[56] was reported[92] but the yields were low because the equilibrium between the pregnanes[57] and 17-isopregnanes[56] lies well towards the former.

The synthesis of 17-epitestosterone[49] devised by Sondheimer et al.[88] [diagram 1] involved a new route to Δ^{16}-androstene derivatives since previous routes to these compounds required the 17α-ols as starting materials. Dehydroepiandrosterone acetate[58] was converted to its cyanohydrin derivative, which was dehydrated and hydrolysed under alkaline conditions to yield 3β-hydroxy-$\Delta^{5,16}$-etiadienic acid[59]. This compound was decarboxylated to the $\Delta^{5,16}$-dien-3β-ol[60] by refluxing with quinoline and copper chromite, and oxidation under Oppenauer conditions gave the $\Delta^{4,16}$-dien-3-one[61]. Perbenzoic acid oxidation occurred preferentially at the D-ring to yield the 16α,17α-oxide[62] which was reduced with lithium aluminium hydride to the 3β,17α-diol[63]. Oxidation with manganese dioxide gave 17-epitestosterone[49] in 42% yield from the dienol[60].

17-Methylepitestosterone[50] was synthesised[88] by an analogous route based on the decarboxylation of 3β-hydroxy-pregna-5,17(20)-dien-21-oic acid[64] which was prepared from

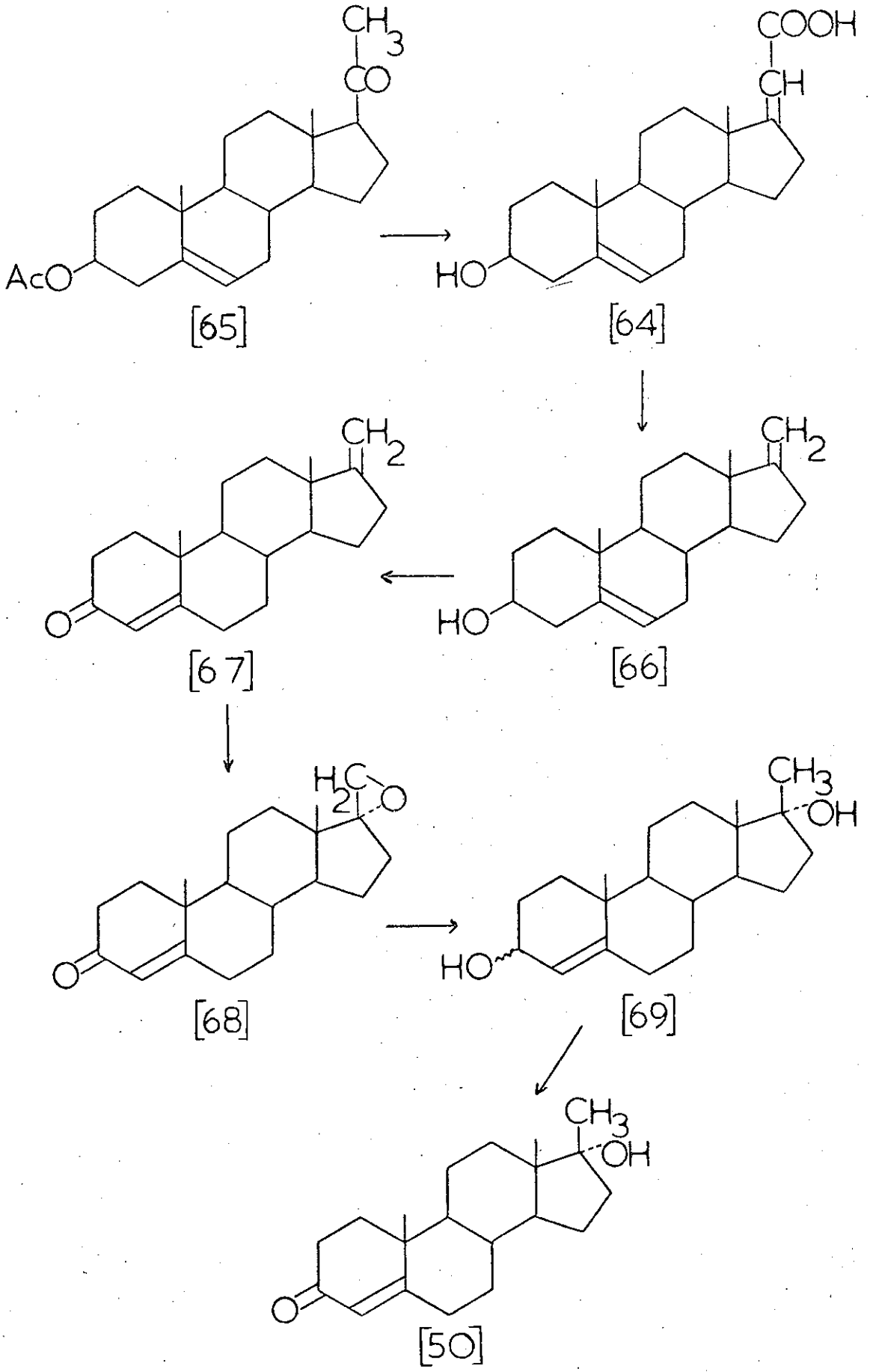


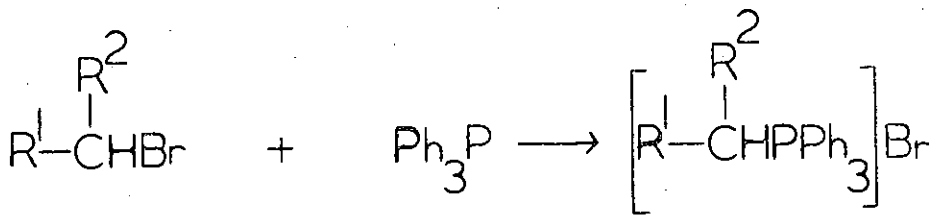
Diagram 2. Previous synthesis of 17-methylepitestosterone.

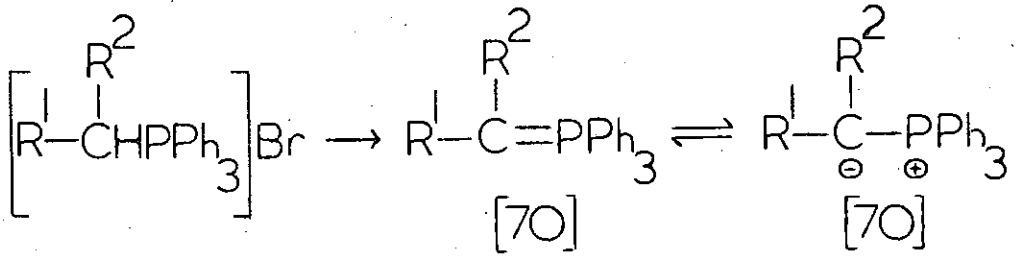
3β-hydroxypregn-5-en-20-one acetate[65] by bromination, reaction with sodium iodide and a Favorskii rearrangement. The 17-methylene-5-en-3β-ol[66] was obtained by refluxing with quinoline in the presence or absence of copper chromite. Oppenauer oxidation gave the 4-en-3-one[67] which was oxidised with perbenzoic acid to the 17α,20-oxide[68]. Lithium aluminium hydride reduction gave the 17β-methyl-17-ol[69] which was oxidised with manganese dioxide to give 17-methylepitestosterone[50]. 17-Ethylepitestosterone has been prepared by a similar route.[93]

3.1.iii. 17-Methyleneandrost-5-en-3β-ol[66]

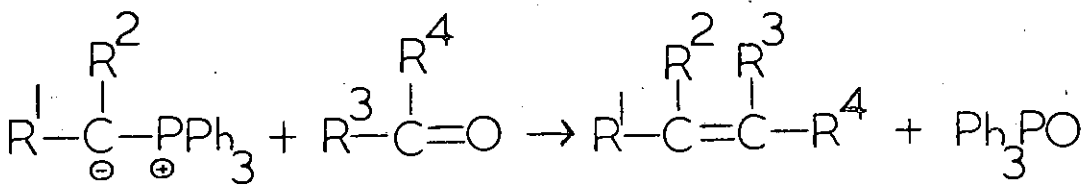
The discovery[94,95] of the reaction between alkylidene phosphoranes[70] and carbonyl compounds[71] simplified the synthesis of 17-methylepitestosterone by offering an alternative route to the 17-methylene compound[66].

Sondheimer and Mechoulam[96] first investigated the use of the Wittig reaction for the synthesis of steroidal methylene compounds and reported that the reaction proceeded most smoothly when methylenetriphenylphosphorane[70, $R^1=R^2=H$] was employed. The ylide was generated in ethereal solution with butyllithium as the base according to the procedure described by Wittig.[94,95] A solution of the steroidal ketone in ether was added and the reaction carried out under reflux in tetrahydrofuran after removal of the ether by distillation. Reaction of 3β-hydroxy-5α-androstan-17-one with 3 equivalents of the Wittig reagent gave a 32% yield of the corresponding 17-methylene compound. When the quantity of Wittig reagent was increased to 5 equivalents a 58% yield was obtained; consequently a very

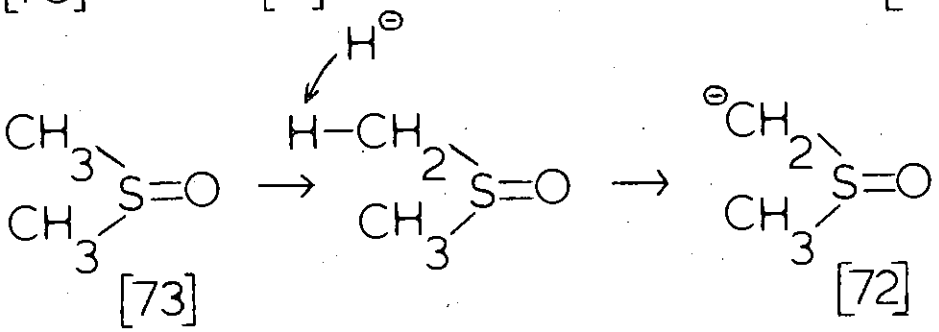




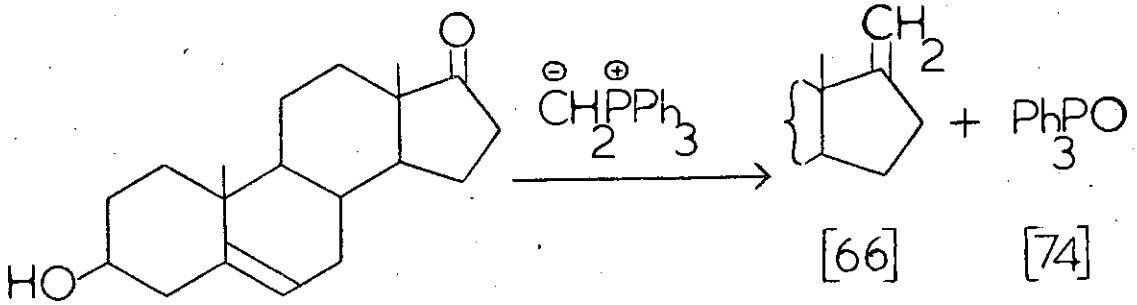
[70] [70]



[70] [71] [74]



[73] [72]



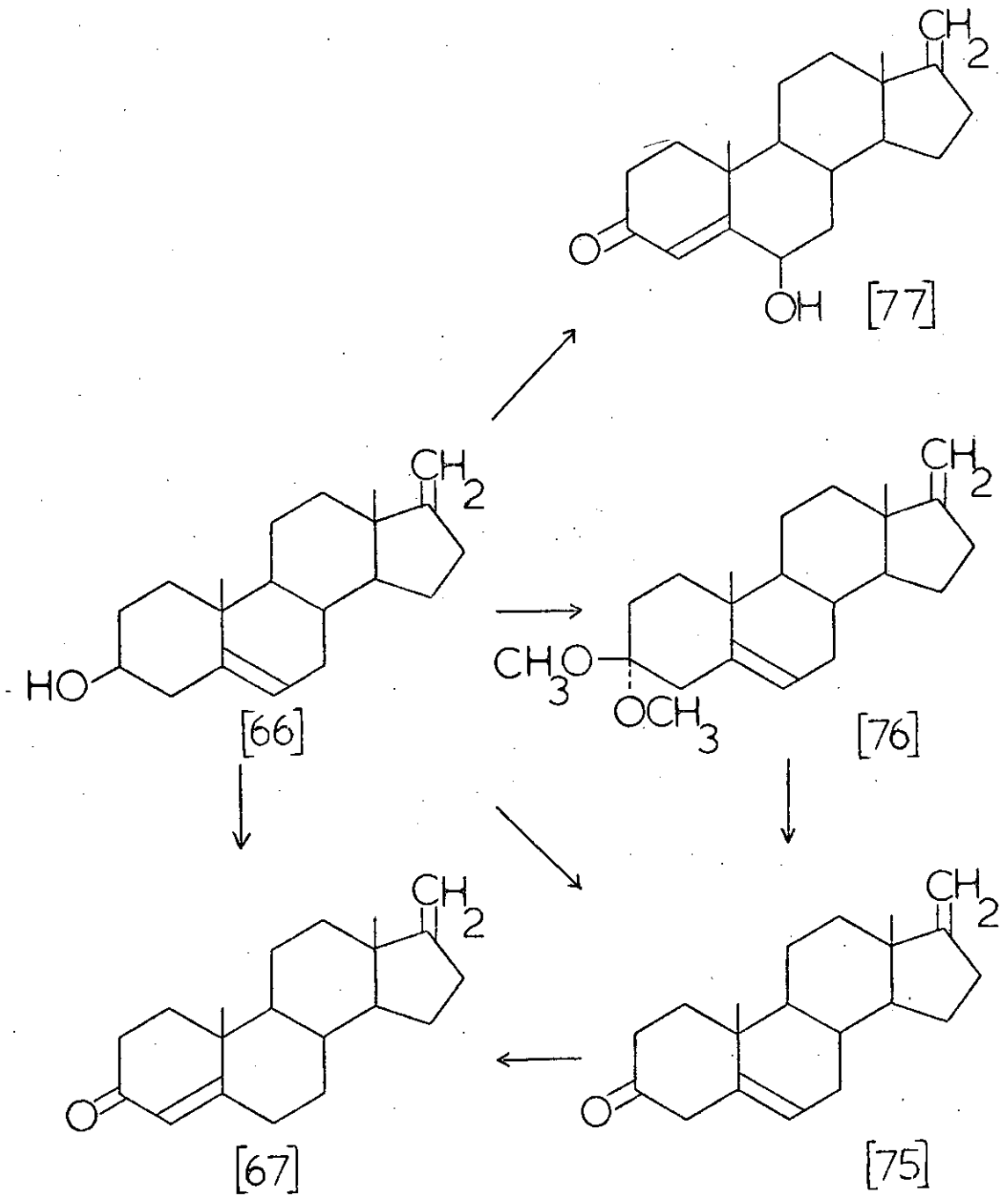
[48] [66] [74]

considerable excess of reagent was recommended for the reaction of polyfunctional compounds. Treatment of dehydroepiandrosterone[48] with 3 equivalents of the reagent gave the 17-methylene derivative[66] in 36% yield. The effect of protection of the 3β-hydroxyl group on the yield was investigated but the free ketol proved to be the most suitable starting material.[96]

In 1962 Corey and Chaykovsky[97] reported the preparation of methylsulphinyl carbanion[72] from the reaction of sodium hydride and dimethyl sulphoxide[73]. Greenwald *et al.*[98] used this base for the preparation of the Wittig reagent[70] and found that both the rate and yield of the overall reaction were greatly improved in comparison to those of the reaction in customary solvents. Potassium t-butoxide was introduced for the generation of methylsulphinyl carbanion by Drefahl *et al.*,[99,100] who obtained 17-methyleneandrost-5-en-3β-ol[66] in 80% yield.

In their synthesis of the 17-methylene compound[66] Drefahl *et al.*[100] maintained the mixture of steroid and ylide solutions at room temperature for 12 hours before heating at 80°C for 15 hours and subsequent extraction of the product. Several variations of this procedure have now been tested and thin layer chromatography of the reaction mixture showed that all the starting material[48] was consumed after the solutions were mixed, heated directly to 80°C and maintained at this temperature for 1 hour. Rigorous exclusion of water was necessary for successful formation of the ylide.

The presence of the byproduct of the reaction, triphenylphosphine oxide[74], in the crude product was easily detected


CH2
O
OH
[77]
HO
[66]
CH3O
OCH3
[76]
[67]
[75]

by intense absorptions at ν_{max} 1210, 1125 and 730cm^{-1} in the infrared spectrum. Alumina chromatography has been used[96,100] to purify the product but several recrystallisations from methanol proved more convenient and consistently gave 75-80% yields of pure compound, judged by the absence of the triphenylphosphine oxide bands in the infrared spectrum. The infrared and n.m.r. spectra contained bands at ν_{max} 1650 and 895cm^{-1}[101] and a two proton signal at τ5.36 respectively, characteristic of a terminal methylene function. When acetone was employed as recrystallising solvent co-crystals of triphenylphosphine oxide and the steroid[66], which melted at a similar temperature to the pure steroid, were obtained.

3.1.iv. 17-Methyleneandrost-4-en-3-one[67]

The Oppenauer oxidation,[102] used by Sondheimer et al.[88] converted the Δ^5-en-3β-ol[66] to the 4-en-3-one[67] in a single step, although it probably involved initial formation of the β,γ-unsaturated ketone[75] which would be immediately isomerised in the basic medium.[103] This reaction proved unsuitable because of the difficulty of freeing the product[67] from traces of cyclohexanone and its condensation products, which resulted in large losses of steroid. Consequently, the Δ^5-en-3β-ol[66] was oxidised to the Δ^5-en-3-one[75] with chromic acid in acetone.[103,104] Mineral acid has been used[103] to isomerise the 5,6-double bond to the conjugated 4,5-position but when this reagent was employed much degradation occurred. However anhydrous oxalic acid[105] proved successful for the isomerisation.

In an attempt to minimise degradation of the steroid

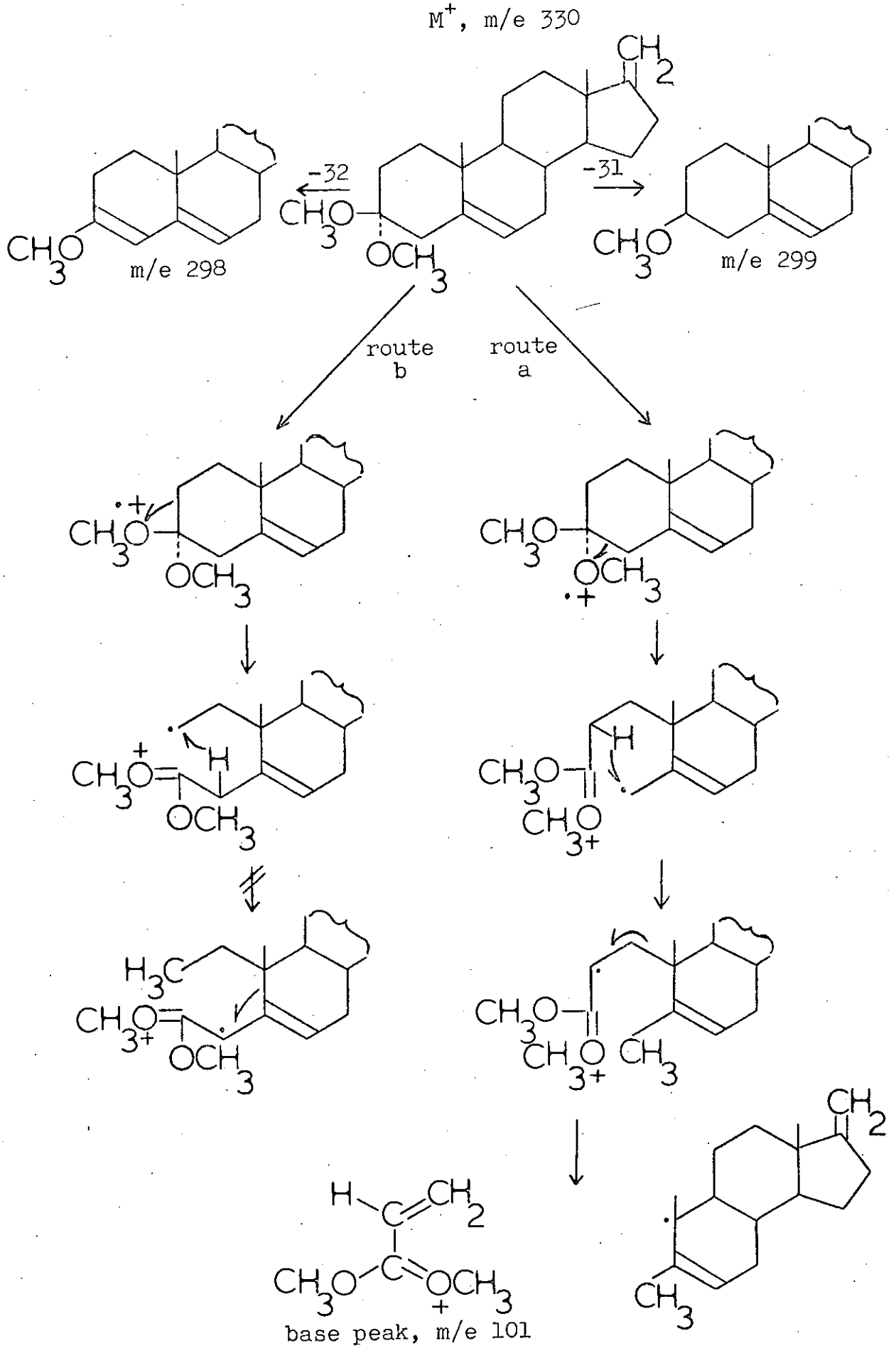


Diagram 3. Mass spectral fragmentations of 17-methyleneandrost-5-ene-3-dimethyl ketal.

during removal of the organic solvents after chromic acid oxidation saturated sodium bicarbonate solution was added immediately after the reaction had been quenched with methanol. The solvents were then removed and the product extracted by the usual procedure. Infrared analysis of the crude product showed a very low intensity β,γ-unsaturated carbonyl absorption, ν_{max} 1715cm^{-1}, in comparison to the C-H stretch at ν_{max}2990-2910cm^{-1} and several intense bands in the fingerprint region not found in the spectrum of the Δ^5-en-3-one[75]. N.m.r. spectroscopy indicated that the double bond was entirely in the 4,5-position (τ4.64,6-H) and that the 17-methylene function was intact (τ5.36). The shift of the C-19 methyl signal (τ8.98) compared to that of the Δ^5-en-3-one[75] (τ9.17) was consistent with the assignment of two singlets (τ6.89, 6.80) to a 3-dimethyl ketal[76].

Alumina chromatography of the crude product led to almost immediate elution of a white crystalline compound, the infrared and n.m.r. spectra of which showed the features described above. Zalkow *et al.*[106] reported signals at τ6.86 and 6.81 in the n.m.r. spectrum of 5α-cholestane-3-dimethyl ketal. These authors stated that the reaction medium should be absolutely anhydrous to obtain a high degree of ketal formation, however the n.m.r. spectrum showed that the crude product contained about 60% of the ketal[76].

The mass spectrum of the low polarity product taken from the column confirmed that it was 17-methyleneandrost-5-ene-3-dimethyl ketal[76]. The ethylene ketal, which is a common protective group for steroid ketones, produces such

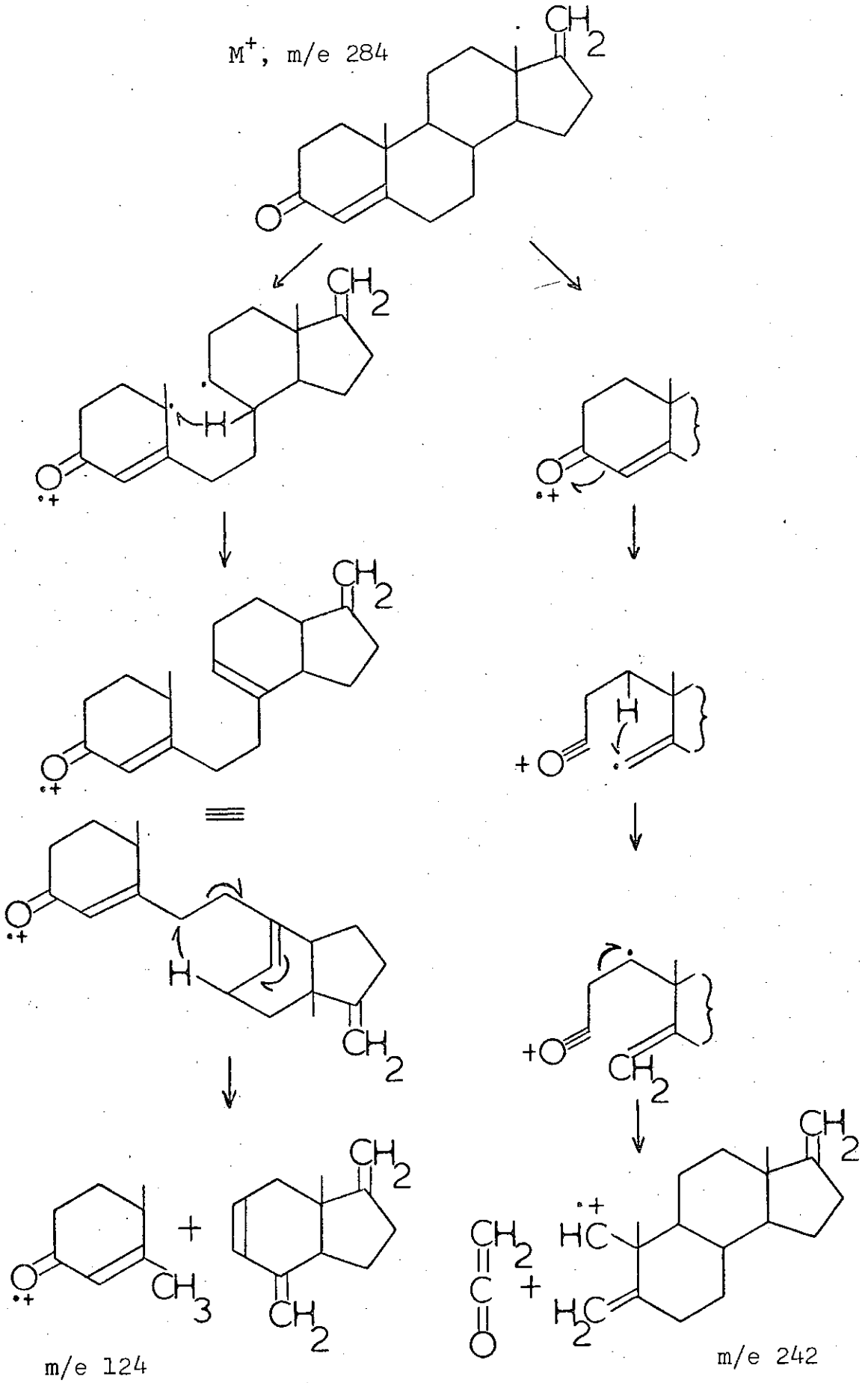


Diagram 4. Mass spectral fragmentations of 17-methyleneandrost-4-en-3-one.

pronounced fragmentation that it overshadows the effects of other functional groups.[107a] The dimethyl ketal function in 17-methyleneandrost-5-ene-3-dimethyl ketal acted in the same way and gave rise to the base peak at m/e 101 by the route shown in diagram 3. As in the case of the ethylene ketal of androst-5-en-3-one[107a] the 5,6-double bond favours cleavage of the 3,4-bond by allylic activation [route a] whereas an ion cannot arise from 2,3-bond cleavage [route b] because of the unfavourable allene intermediate which would be involved. The only other ions of importance in the mass spectrum of the ketal were the molecular ion (m/e 330, 9%) and those at m/e 299 (18.5%) and m/e 298 (22%). These two latter ions arise from loss of a methoxy radical and the elements of methanol, respectively.

Approximately 40% of the crude product was eluted from the column as the ketal[76]. However, the next component was identified as the Δ^4-en-3-one[67] (12%) which could have arisen only by hydrolysis of the ketal and conjugation on the alumina. It was identical with the compounds obtained by Oppenauer oxidation of the Δ^5-en-3β-ol[66] and by oxalic acid isomerisation of the Δ^5-en-3-one[75]. The mass spectrum of 17-methyleneandrost-4-en-3-one was more complex than that of the dimethyl ketal[76]. The molecular ion (m/e 284) was also the base peak and the spectrum included peaks at m/e 242[108] and m/e 124[109] characteristic of the Δ^4-en-3-one system[107b] [see diagram 4].

Unreacted Δ^5-en-3β-ol[66] was next eluted from the column. The most important ions in the mass spectrum of

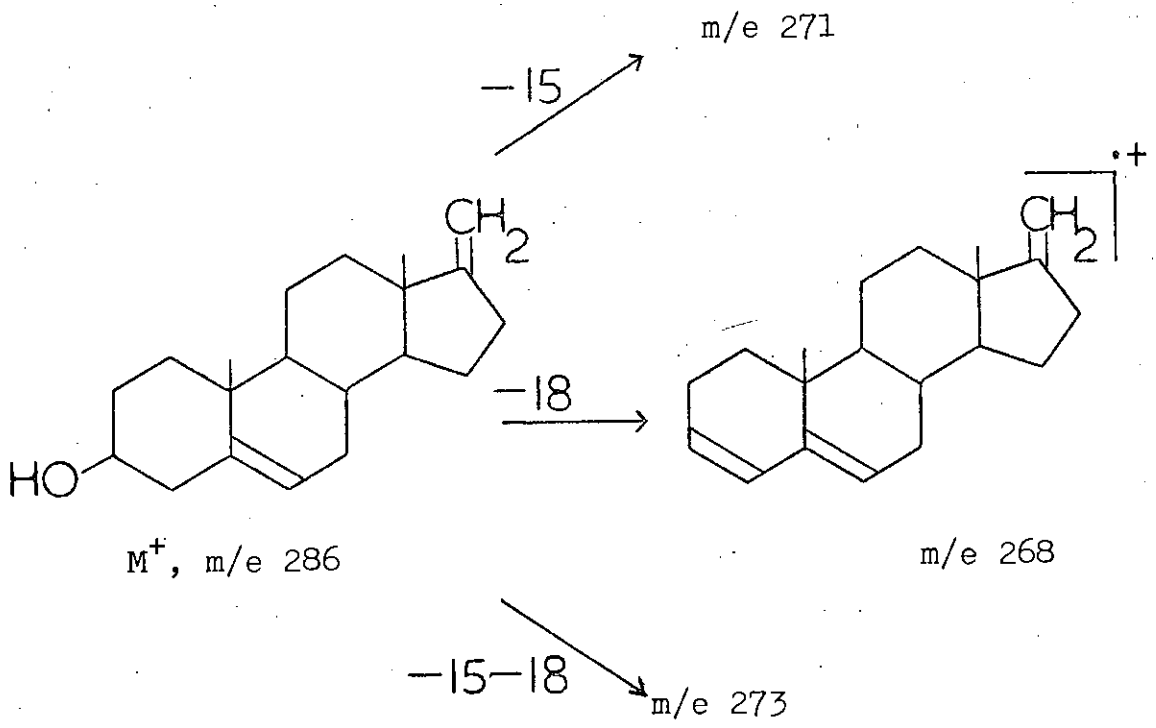


Diagram 5. Mass spectral fragmentations of 17-methyleneandrost-5-en-3β-ol.

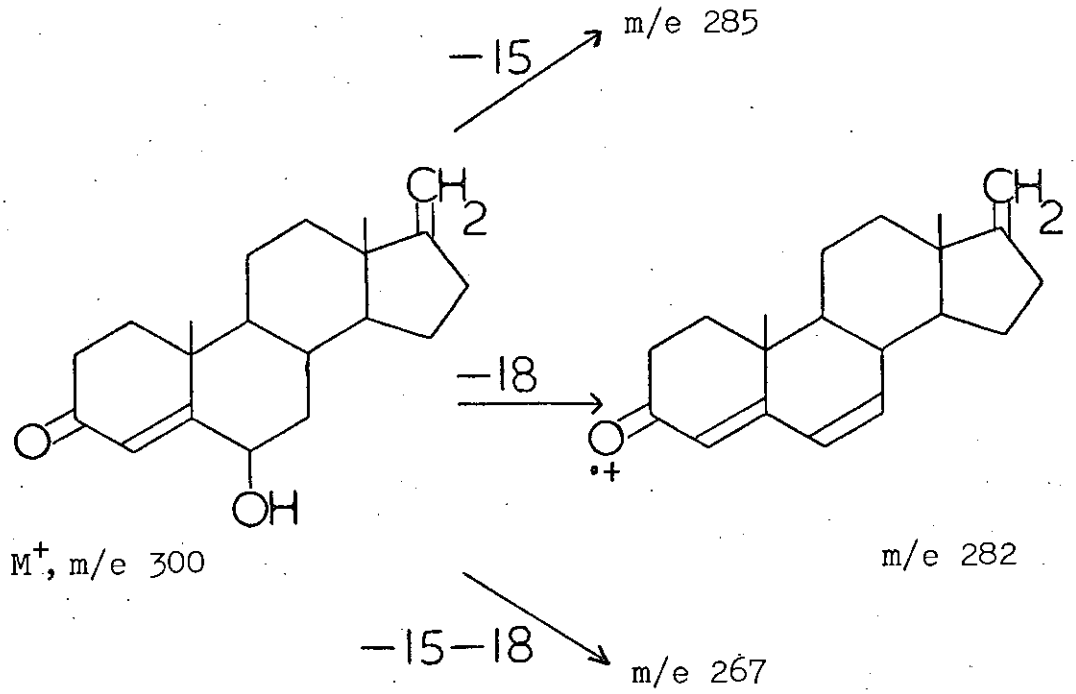
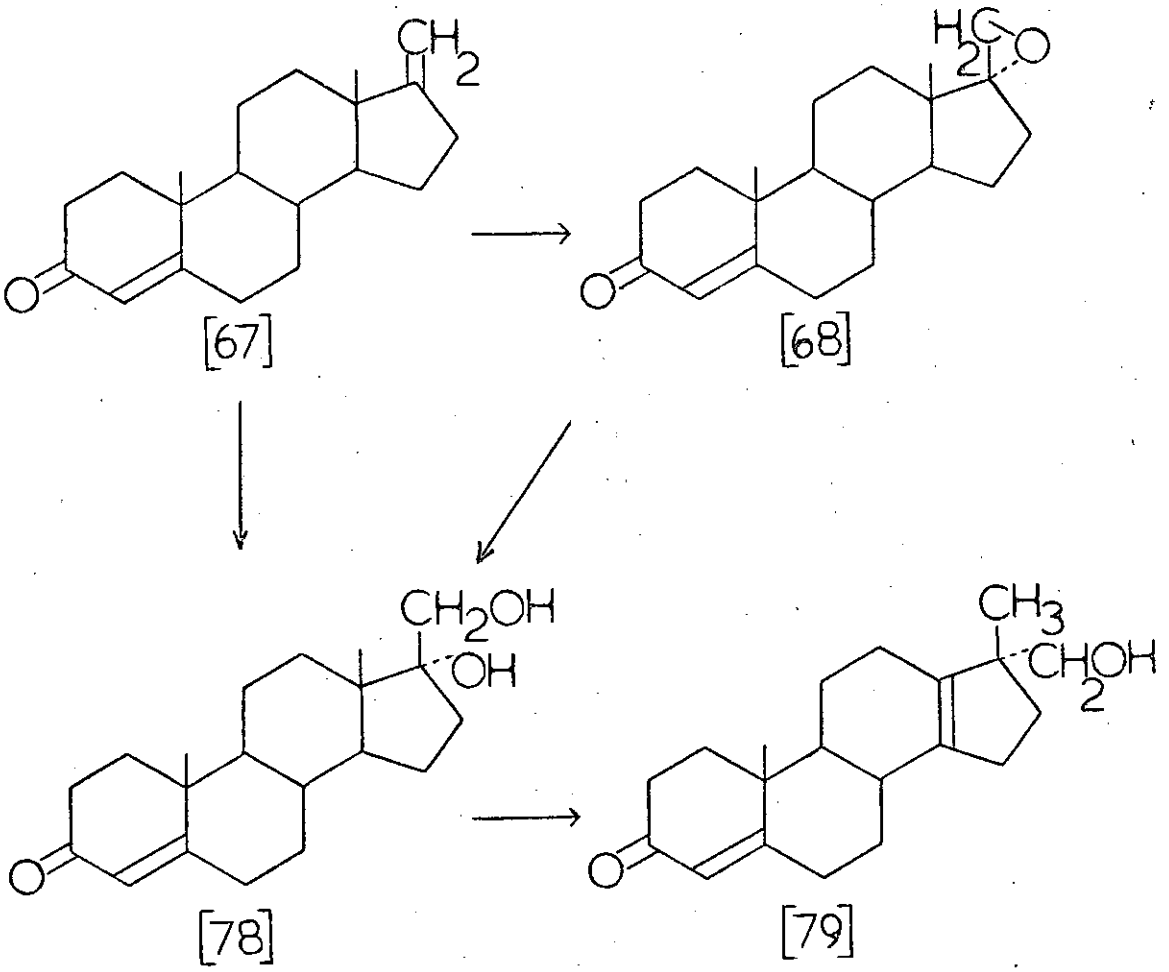


Diagram 6. Mass spectral fragmentations of 6β-hydroxy-17-methyleneandrost-4-en-3-one.

this compound [see diagram 5] were the molecular ion (m/e 286), which was also the base peak, and ions at m/e 271, m/e 268 and m/e 253, which arose from loss of a methyl radical, water and the two moieties together, respectively.

A second unknown product was then eluted, the infrared spectrum of which showed that it contained a hydroxyl group (ν_{max}3500cm^{-1}) in addition to the 17-methylene function. The conjugated carbonyl absorption (ν_{max}1680cm^{-1}) was slightly different from that of 17-methyleneandrost-4-en-3-one (ν_{max}1675cm^{-1}). As well as the C-4 proton signal, which was shifted downfield by 3Hz compared to the corresponding signal in the Δ^4-en-3-one [67], and the 17-methylene resonance, the compound gave a single proton triplet at τ5.65 (J = 3Hz). The 18-methyl shift (τ9.16) was similar to that of the other 17-methylene steroids encountered but the 19-methyl resonance (τ8.54) was at considerably lower field. Similarly the n.m.r. spectrum[110] of the known 6β-hydroxyandrost-4-en-3-one has a triplet at τ5.65 (J = 3Hz) corresponding to the 6α-proton and the 19-methyl signal is low (τ8.62). The molecular ion of the unknown product (m/e 300), which was also the base peak, was consistent with one additional hydroxyl group [see diagram 6]. The only other intense ions in the mass spectrum were those resulting from loss of a methyl group, water and both at m/e 285, m/e 282 and m/e 267, respectively. The compound was thus identified as 6β-hydroxy-17-methyleneandrost-4-en-3-one [77]. 6β-Hydroxycholest-4-en-3-one has been reported


CH2
O
[67]
H2C
O
[68]
CH2OH
OH
O
[78]
CH3
CH2OH
O
[79]

to be among the products of the dichromate oxidation of cholesterol.[111a]

Mild acid hydrolysis of the ketal [76] gave a mixture of the enones [75,67] which was isomerised to the Δ^4-en-3-one[67] with oxalic acid.

3.1.v. 17α,20-Oxido-21-norpregn-4-en-3-one[68]

Sondheimer et al.[88] used perbenzoic acid for the conversion of 17-methyleneandrost-4-en-3-one[67] to the 17α, 20-oxide[68]. More recently epoxidising agents, such as m-chloroperbenzoic acid, which remain active for long periods when stored at room temperature, have been developed. m-Chloroperbenzoic acid, manufactured by the FMC Corporation, was used for the first epoxidations of the 17-methylene compound, according to a method developed by the manufacturers.[112a] The reagent had a minimum assay of 80% active oxygen when produced but at the time of use the assay was 60%, determined by iodimetric titration.

The products of the epoxidation reaction were examined most conveniently by thin layer chromatography and n.m.r. spectroscopy. After development of the plate with sulphuric acid spray and heat the epoxide [68] was readily recognisable as a greenish-yellow spot, slightly more polar than the initially pink spot of the starting material [67] and considerably less polar than the blue spot given by 17α-hydroxy-17-hydroxymethylandrost-4-en-3-one [78]. Epoxide formation was easily detected by n.m.r. spectroscopy by the disappearance of the 17-methylene signal (τ5.36) and the appearance of a pair of doublets at τ7.34, 7.26 (J_{AB} = 4Hz) assigned to

the protons of the 20-methylene function. The C-20 protons of the glycol[78] gave a pair of doublets at τ6.43, 6.38 (J_{AB} = 11Hz).

When the epoxidation was carried out at room temperature for periods of between 1 and 3 hours unreacted starting material[67] was isolated at reaction concentrations of ca. 0.0024 moles of active oxygen per 0.0036 moles of steroid per litre of methylene chloride but at concentrations of 0.136 moles of active oxygen per 0.136 moles of steroid per litre of solvent the major product was the glycol[78], formed by cleavage of the epoxide under excessively acid conditions. However, at concentrations in the region of 0.07 moles of active oxygen per 0.08 moles of steroid per litre good yields of the epoxide[68], only slightly contaminated with the unreacted 17-methylene compound[67] and the glycol[78], were obtained. Purification was unnecessary at this stage because the products of reduction of these contaminants were easily removed after the following reaction.

The epoxidation was repeated using new batches of m-chloroperbenzoic acid manufactured by either B.D.H. Chemicals Ltd. or by Ralph N. Emmanuel Ltd. The heat of reaction was now sufficient to boil the solvent and a fluorescent green-red colour developed. The product of epoxidation under these conditions contained a mixture of the starting material [67] and the two hydroxymethyl compounds [78,79], but no epoxide[68]. There was no improvement when the temperature was maintained below 25°C during the addition of the peracid and at ice temperature the reaction did not proceed at all.

A colour change similar to that described above was noted in an experiment, described later (section 3.15.ii), in which the glycol[78] was heated with sulphuric acid. Dehydration and methyl migration again occurred to give 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one [79], the hydroxymethyl protons of which gave a pair of doublets at τ6.70, 6.60 (J_{AB} = 10Hz).

Titrimetric analysis of the fresh peracids showed that the average active oxygen content was 86%, in comparison to the 60% activity of the older reagent. Since the diluent in this latter material might have been the parent acid, fresh peracid was mixed with m-chlorobenzoic acid to give a sample with 60% activity. However, this reagent also gave overreaction under the previous conditions.

Ruzicka et al.[113] obtained at least three different products from the oxidation of 17-ethylideneandrost-4-en-3-one with 1.5 equivalents of monoperphthalic acid (0.088 mole of active oxygen/0.057 mole of steroid/litre) but oxidation with 0.9 equivalents of perbenzoic acid (0.237 mole of active oxygen/0.264 mole of steroid/litre) gave predominantly the 17α,20-epoxide.[88] Reaction of 0.9 equivalents of perbenzoic acid, prepared according to the method of Moyer and Manley,[114] with 17-methyleneandrost-4-en-3-one (0.024 moles of active oxygen/0.027 moles of steroid/litre) for 2-3 hours gave a mixture of the epoxide[68](35%), unreacted starting material[67](50%) and the glycol[78](15%). A longer reaction time led to complete degradation of the epoxide.

Satisfactory yields of 17α,20-oxido-21-norpregn-4-en-

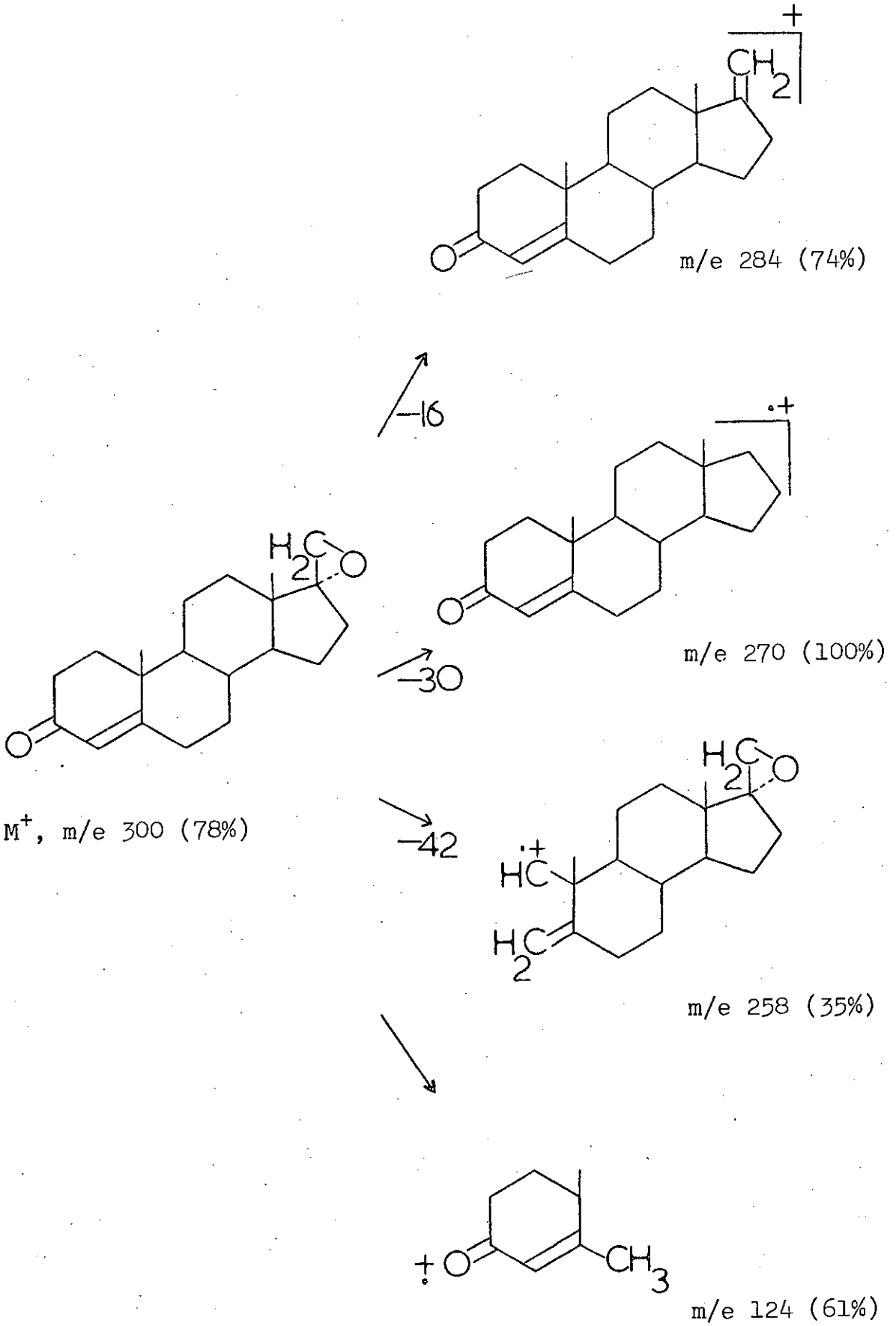


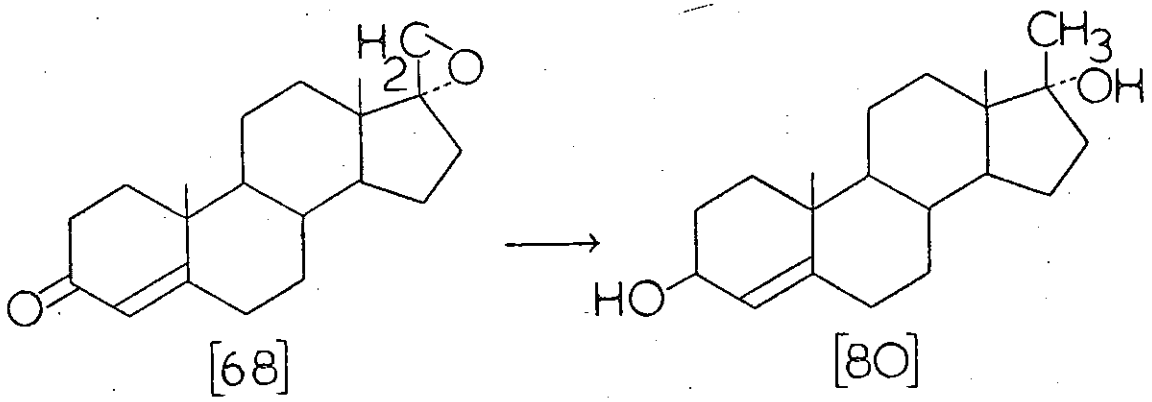
Diagram 7. Mass spectral fragmentation of 17α,20-oxido-21-norpregn-4-en-3-one.

3-one, contaminated with some unreacted starting material but with negligible glycol were finally achieved with the 86% active m-chloroperbenzoic acid at concentrations of ca. 0.019 moles of active oxygen per 0.018 moles of steroid per litre of solvent.

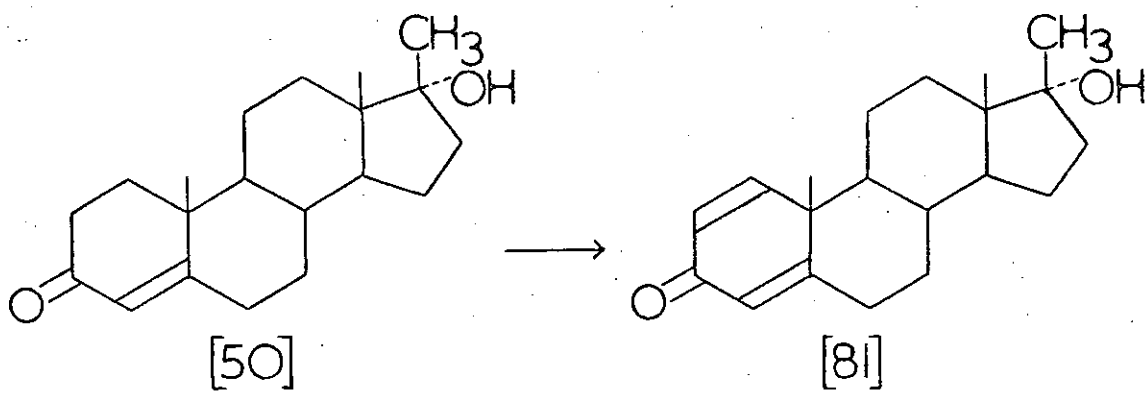
The mass spectrum of the epoxide [68] [see diagram 7] contained the expected molecular ion at m/e 300 together with intense ions arising from loss of the epoxide oxygen atom (m/e 284) and of the epoxide function (m/e 270, base peak). Other important ions in the spectrum were those characteristic of the Δ^4-en-3-one system (m/e 258 and m/e 124).

3.1.vi. 17β-Methylandrost-4-en-3β,17-diol [80]

Sondheimer et al.[88] reported the formation of a mixture of the C-3 epimeric 17β-methylandrost-4-ene-3,17-diols by lithium aluminium hydride reduction of the 17α,20-epoxide [68]. Reduction of an unhindered C-3 carbonyl group has been shown to give approximately 90% of the equatorial 3β-alcohol in most cases,[115] because of attack from the less crowded α-face of the steroid molecule. However, α,β-unsaturation can have a profound effect on the stereochemistry of reduction, as illustrated by cholest-4-en-6-one and cholest-5-en-4-one, both of which have been reduced[116] to the α-alcohols. These results are explained[116] by distortions caused by the double bond, which tend to pull the carbonyl group into the plane of the molecule and so decrease the crowding on the β-face. The choice of solvent and reaction temperature can also affect the stereochemistry of the



[68] [80]



[50] [81]

products of hydride reductions.[117a]

The product of the lithium aluminium hydride reduction of the epoxide[68], which was easily purified by virtue of its poor solubility in acetone in comparison to the reduction products of the 17-methylene compound [67] and the glycol[78], gave only one spot on thin layer chromatography. However, it was doubtful whether a mixture of the 3α- and 3β-alcohols could have been separated in the solvent system employed. The n.m.r. spectrum of the product showed that the double bond was no longer conjugated (τ4.72, 4-H) and a multiplet at τ5.85 ($w_{\frac{1}{2}}$ = 18Hz) was in good agreement with the reported spectrum[110] of androst-4-en-3β-ol (τ5.85, m, $w_{\frac{1}{2}}$ = 15Hz, 3α-H). Since the 3β-proton of androst-4-en-3α-ol resonates[110] at τ5.91 (m, $w_{\frac{1}{2}}$ = 10Hz) it was impossible to determine, from this region of the spectrum, whether the product contained any of the 3α-ol because of the band width of the 3α-proton and the poor solubility of the sample. The 19-methyl signal of the product (τ8.95) was similar to that of androst-4-en-3β-ol (τ8.93[110]) but there was no evidence of a 19-methyl signal similar to that of androst-4-en-3α-ol (τ9.01[110]). Consequently the product of the lithium aluminium hydride reduction of 17α,20-oxido-21-norpregn-4-en-3-one was assumed to be 17β-methylandrost-4-ene-3β,17-diol[80]. Oppenauer oxidation of the enediol [80] gave 17-methylepitestosterone[50] which was reduced with sodium borohydride[118] to give a product which was predominantly the 3β-ol[80] but also contained a small amount of the epimeric 3α-ol (C-19 methyl, τ9.01).

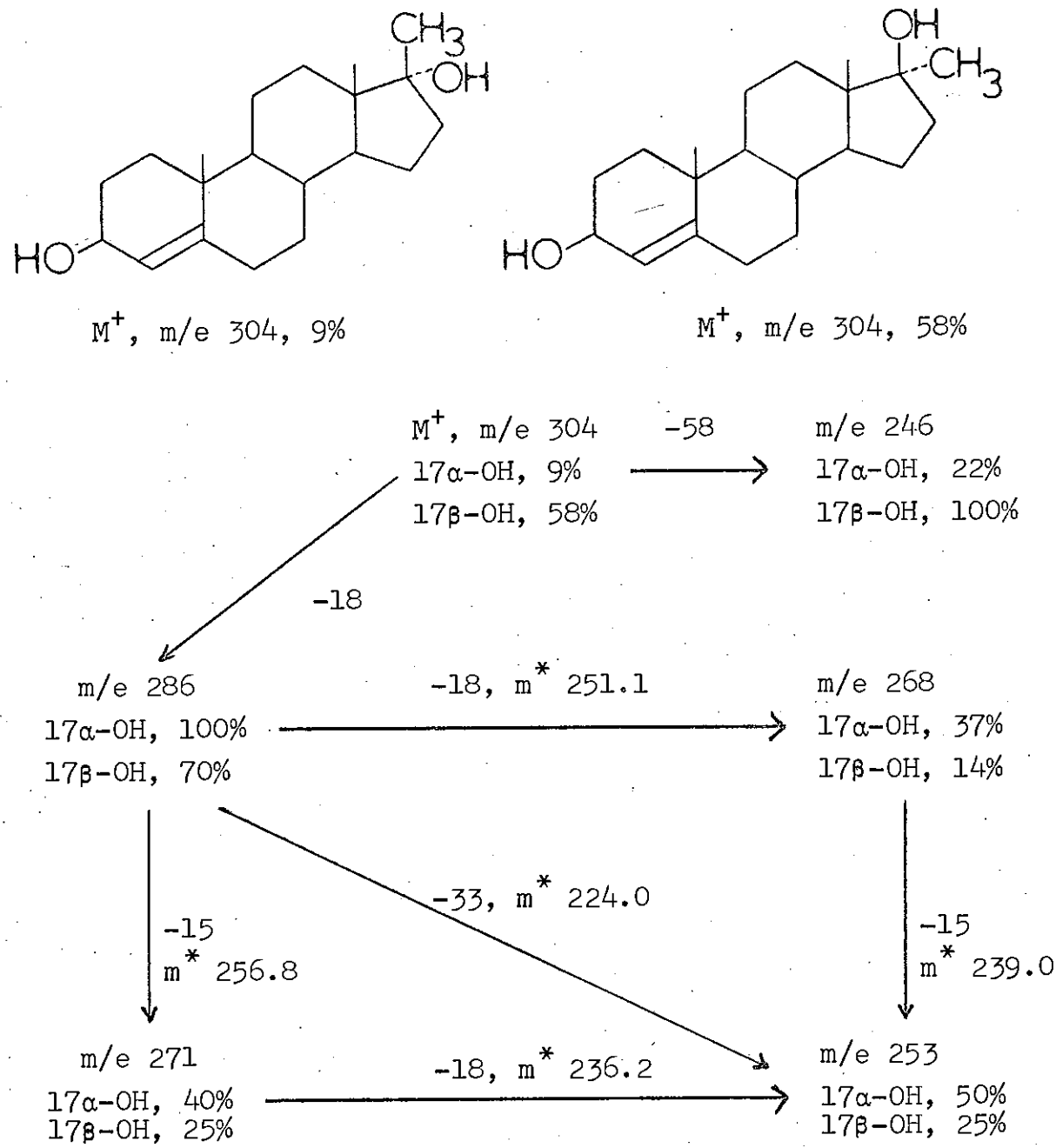


Diagram 8. Mass spectral fragmentations of the epimeric 17-methylandrost-4-ene-3β,17-diols.

The mass spectrum of 17β-methylandrost-4-ene-3β,17-diol [see diagram 8] contained a very low intensity molecular ion (M^+, m/e 304), the base peak arising from loss of water from the molecular ion (m/e 286). Metastable peaks (m^*) showed that the base peak lost a methyl radical and water by the three pathways shown in diagram 8. 17α-Methylandrost-4-ene-3β,17-diol was obtained by sodium borohydride reduction[118] of methyltestosterone and its mass spectrum contained the same ions as that of its C-17 epimer. However the intensities of the characteristic ions relative to the base peak in each spectrum were quite different [diagram 8].

3.1.vii. 17-Epimethandrostenolone[81]

Muller et al.[119] described the use of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) for the preparation of 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione 21-acetate from the 3-keto-5β- and 3α-hydroxy-5β- steroid precursors. A concentration of 3-4 moles of DDQ per mole of 3α-hydroxy-5β-steroid was recommended for dehydrogenation to the dienone; consequently, since the enediol [80] already contained one of the required A-ring double bonds the quantity of DDQ was reduced to 2.2 moles per mole of steroid.

Alumina chromatography was used to remove several low polarity products of this reaction from the two major products which were of very similar polarity. These compounds were separated into two fractions, each rich in one of the components, by further chromatography. The two pure compounds were obtained by recrystallisation. A single proton resonance at τ4.28 in the n.m.r. spectrum and a carbonyl band at ν_{max}

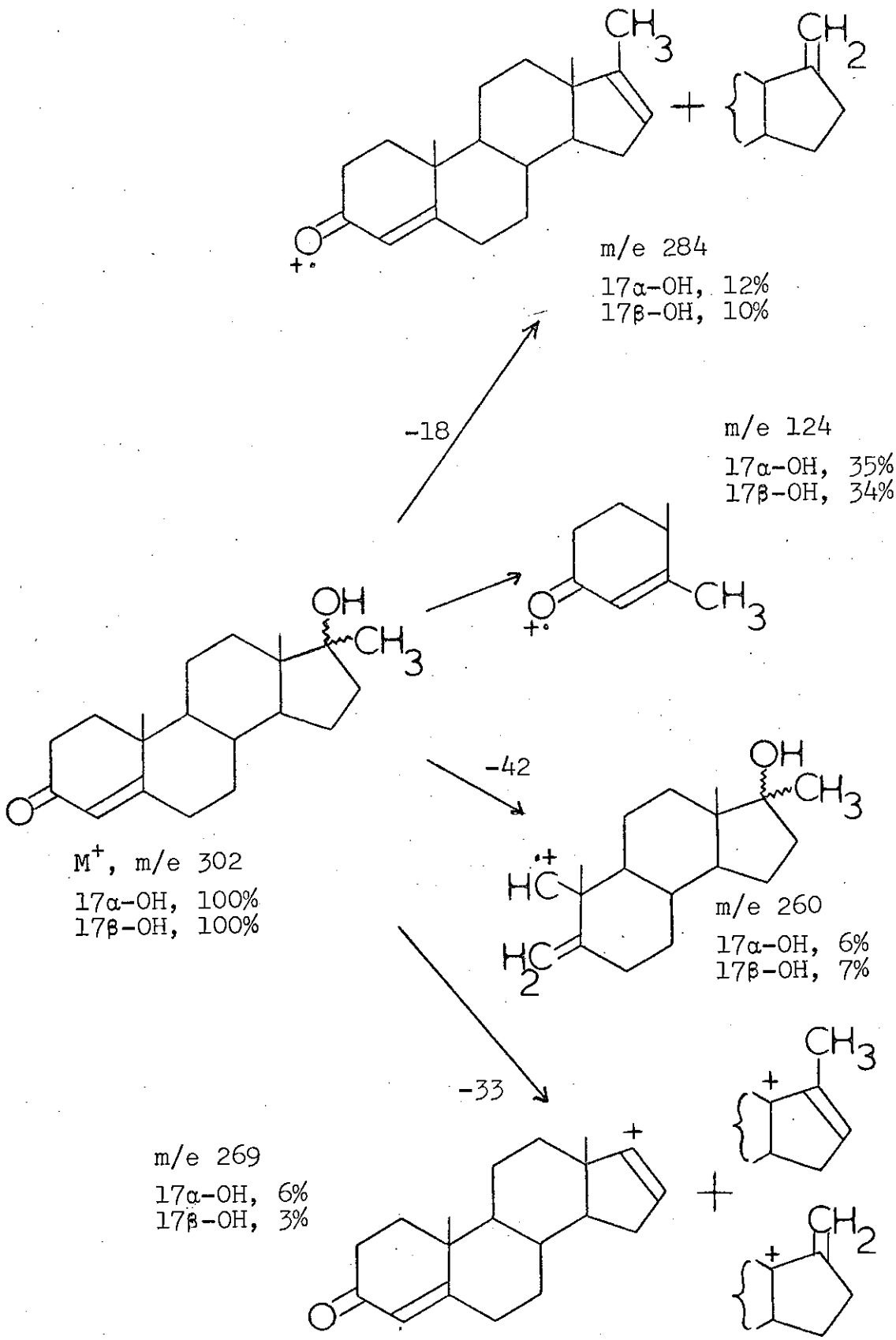


Diagram 9. Mass spectral fragmentations of the epimeric 17-methyltestosterones.

1675cm^{-1} in the infrared spectrum indicated that the less polar product was a Δ^4-en-3-one. Its melting point (180-182°C) was in agreement with the literature melting point[111b] of 17-methylepitestosterone [50] and the mass spectrum (M^+, m/e 302) confirmed the identification of the compound. Diagram 9 illustrates the close similarity between the mass spectrum of this compound and that of its C-17 epimer, methyltestosterone.

The crystalline compound isolated from the most polar fraction was identified as the 1,4-dien-3-one, 17-epimethandrostenolone[81]. The properties of this compound and its 17-epimer, methandrostenolone are compared and discussed in section 3.3.

Experimental Procedure

Melting points were determined on a Kofler hot stage apparatus and are corrected. Infrared spectra of carbon disulphide solutions were recorded on a Unicam S.P. 200 spectrometer and ultraviolet spectra were recorded on a Unicam S.P. 800 spectrometer. Nuclear magnetic resonance spectra of deuteriochloroform solutions were recorded on a Perkin Elmer R10 (60 M Hz) spectrometer or a Varian HA 100 (100 M Hz) spectrometer with tetramethylsilane as an internal standard. Optical rotations were measured on a Perkin Elmer model 141 automatic polarimeter at 589nm in methanol solutions. Analyses were determined on a Perkin Elmer model 240 instrument.

Merck silica gel GF_{254} was used for thin layer chromatography and the eluting system was benzene-ethanol (9:1) unless otherwise stated. The plates were developed with sulphuric acid in ethanol (1:19) spray and then heated. Alumina refers to Spence type H alumina of Brockman activity 2 and 5% deactivated alumina to alumina deactivated with 5% by volume of a 10% solution of acetic acid.

Mass spectrometry was carried out on an AEI MS 902 instrument operated at 70eV by Mr. D. Thomas. Microgram samples for mass spectrometry were weighed on a Cahn 1400 range selector electrobalance. Very small samples, particularly of metabolites, were transferred to a quartz direct insertion probe as solutions in chloroform and the solvent removed by evaporation.

Gas liquid chromatography was carried out on a Perkin Elmer 801 instrument with 6 ft. all-glass columns of $\frac{1}{8}$"

internal diameter and a Pye 104 instrument with 6 ft. all-glass columns of ¼" internal diameter. Stationary phases were the methylsilicones, OV-1 and QF-1 and the methyl-siloxane polymer, SE-30. Various column temperatures between 220 and 250°C were used and are stated in the text. The detector and injector were maintained at slightly higher temperatures than the column. Preparative gas chromatography was carried out with the aid of a column splitter over the end of which a 6" length of glass tubing (5mm internal diameter) was placed. Samples condensed approximately half way along the glass tube and were located as a slight mist covering a ½" band. Both ends of the tube were removed to leave a section (ca. 1") containing the steroid which was then eluted, evaporated to dryness and redissolved in a small volume of solvent. This sample was either rechromatographed or transferred to a direct insertion probe for mass spectrometry.

Methandrostenolone and oxymetholone were extracted from commercial tablets of the respective drugs by first grinding the tablets to a fine powder with a pestle and mortar. The powder was transferred to a flask and benzene was added. After periodic shaking of the mixture during ca. 30 minutes the insoluble carrier material was removed by filtration and the filtrate was evaporated to dryness to give the pure steroid.

3.2 Experimental Section

3.2.i. 17-Methyleneandrost-5-en-3β-ol [66][100]

Methyltriphenylphosphonium bromide[120] (25.0g) and potassium t-butoxide:t-butanol[112b] (1:1 complex, 13.04g) were dissolved in dry dimethyl sulphoxide (80ml) under nitrogen with gentle warming. A solution of 3β-hydroxyandrost-5-en-17-one (4.04g) in dimethyl sulphoxide (80ml) was added. The reaction mixture was maintained at 80°C for 1 hour, diluted with ice and 10% sodium chloride solution and extracted twice with ether. The ether extracts were combined, washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give the crude product (5.09g), heavily contaminated with triphenylphosphine oxide. Recrystallisation from methanol yielded pure 17-methyleneandrost-5-en-3β-ol (3.17g, 79%); m.p. 134-135° (lit.,[88] 133-134°); ν_{max} 3450, 1655, 895cm^{-1}; n.m.r. (60 M Hz) τ9.20 (C-18 methyl), 8.98 (C-19 methyl), 6.45 (3α-H), 5.36 (17=CH_2), 4.64 (6-H).

3.2.ii. 17-Methyleneandrost-5-en-3-one [75][103,104]

17-Methyleneandrost-5-en-3β-ol (3.07g) was dissolved in ice-cold acetone (215ml), maintained at 0°, and 8N chromic acid (6.14ml, Jones reagent) was added with rapid stirring. After 45 seconds the reaction was quenched with methanol (120ml), water was added, the organic solvents were removed on a rotary evaporator and the steroid was extracted twice into ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to give a crude product (2.83g). Crystal-

lisation from ethanol gave 17-methyleneandrost-5-en-3-one (0.898g, 29.5%); m.p. 109-117°; ν_{max} 1715, 1685, 1655, 895cm^{-1}.

3.2.iii. 17-Methyleneandrost-4-en-3-one[67]

a. Isomerisation of 17-methyleneandrost-5-en-3-one with hydrochloric acid[103]

17-Methyleneandrost-5-en-3-one (0.8g) was dissolved in ethanol to give a pale yellow solution. Gaseous hydrogen chloride was bubbled through for a few seconds and the dark brown solution was left to stand at room temperature for 1 hour. It was then poured into water, extracted with ether and the ether solution washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a brown glass. Crystallisation from acetone-pentane yielded white crystals of 17-methyleneandrost-4-en-3-one (0.3g); m.p. 125-130° (lit.,[88] 129-131°); ν_{max} 1675,1665,1625,895cm^{-1}; n.m.r. (60 M Hz) τ9.17 (C-18 methyl), 8.80 (C-19 methyl), 5.32 (17=C$\underline{H}_2$), 4.23 (4-$\underline{H}$).

b. Isomerisation of 17-methyleneandrost-5-en-3-one with anhydrous oxalic acid[105]

Anhydrous oxalic acid (0.2g) was added to a solution of 17-methyleneandrost-5-en-3-one (0.85g) in ethanol (50ml). The solution was heated at 60° for 30 minutes, water was added and the ethanol was removed by evaporation in vacuo. The steroid was extracted twice into ether, the combined ether extracts were washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium

sulphate and evaporated to dryness. Crystallisation from acetone gave 17-methyleneandrost-4-en-3-one (0.76g) as white needles; m.p. 129-131°(lit.,[88] 129-131°); ν_{max}1675,1655, 1625,895cm^{-1}; n.m.r. (60 M Hz) τ9.17,8.80,5.32,4.23.

c. Isomerisation of 17-methyleneandrost-5-en-3-one via formation of the C-3 dimethyl ketal

17-Methyleneandrost-5-en-3β-ol (1.0g) was dissolved in ice-cold acetone (70ml) and 8N chromic acid (2.0ml) was added to the stirred solution at 0°. The reaction was allowed to proceed for 60 seconds and then quenched with methanol (100 ml). Saturated sodium bicarbonate solution was added dropwise to the stirred mixture until it was approximately neutral. The organic solvents were removed by evaporation in vacuo and the aqueous residue was extracted three times with ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and then with water until all the chromium salts had been removed, dried over anhydrous magnesium sulphate and evaporated to dryness to give a yellow glass; ν_{max}2990-2910(s), 1715(w), 1400-800 (strong bands)cm^{-1}, containing the dimethyl ketal of 17-methyleneandrost-5-en-3-one (60%); n.m.r. (60 M Hz) τ9.20 (C-18 methyl), 8.98 (C-19 methyl), 6.89, 6.80 (s,s,-OC$\underline{H}_3$, -OC$\underline{H}_3$), 5.35 (17=C$\underline{H}_2$), 4.64 (6-$\underline{H}$). The crude product also contained 17-methyleneandrost-5-en-3-one; n.m.r. (60 M Hz) τ9.17 (C-18 methyl), 8.80 (C-19 methyl), 5.35 (17=C$\underline{H}_2$), 4.64 (6-$\underline{H}$). T.l.c. showed that the major product, the ketal, was less polar than the Δ^5-en-3β-ol, Δ^5-en-3-one and Δ^4-en-3-one.

The crude product (1.05g) was dissolved in toluene and chromatographed on alumina (30g). Elution with toluene (150ml) gave white crystals (0.386g, 37%) of 17-methylene-androst-5-ene-3-dimethyl ketal; m.p. 112.5-113.5°(acetone); ν_{max}1655,1110,1065,895cm^{-1}; n.m.r. (60 M Hz) τ9.20 (C-18 methyl), 8.97 (C-19 methyl), 6.89, 6.80 (s,s-OCH_3, $-OCH_3$), 5.37 (17=CH_2), 4.64 (6-H); Mass spectrum, M^+ m/e 330.

Further elution with toluene (150ml) gave a brown gum (6.7mg) which was discarded. The following six fractions eluted with toluene (900ml) gave 17-methyleneandrost-4-en-3-one (0.126g, 12%) as colourless crystals from acetone; m.p. 130.5-135° (lit.,[88] 129-131°); ν_{max}1675,1655,896cm^{-1}; n.m.r. (60 M Hz) τ9.16 (C-18 methyl),8.80 (C-19 methyl), 5.36 (17 = CH_2), 4.27 (4-H); Mass spectrum, M^+ and base peak, m/e 284.

Continued elution with toluene (150ml) yielded a green glass (15.1mg) which was discarded. Two further fractions eluted with toluene gave white crystals of 17-methyleneandrost-5-en-3β-ol (0.228g, 22%); m.p. 124-133°(lit.,[88] 133-134°); ν_{max}1655,895cm^{-1}; n.m.r. (60 M Hz) τ9.20 (C-18 methyl), 8.98 (C-19 methyl), 6.50 (3α-H), 5.36 (17=CH_2), 4.61 (6-H); Mass spectrum, M^+ and base peak, m/e 286.

A further fraction (8.2mg) eluted with toluene (250ml) was discarded, as was the material (31.3mg) eluted with toluene-chloroform (9:1, 500ml). Toluene-chloroform (1:1, 250ml) eluted colourless crystals (43.8mg, 4%) of 6β-hydroxy-17-methyleneandrost-4-en-3-one; m.p. 170-175° (acetone); ν_{max}3500,1680,1655,895cm^{-1}; n.m.r. (60 M Hz) τ9.16 (C-18 methyl),

8.54 (C-19 methyl), 5.67 (t, 3Hz, 6α-H), 5.37 (17=CH_2), 4.22 (4-H); Mass spectrum, M^+ and base peak, m/e 300.

17-Methyleneandrost-5-ene-3-dimethyl ketal (0.30g) was dissolved in acetone (100ml) and dilute sulphuric acid (5ml) was added. The solution was stirred for 1½ hours and then extracted three times with ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a mixture of 17-methyleneandrost-5-en-3-one and 17-methyleneandrost-4-en-3-one; ν_{max} 1715,1675cm^{-1}; n.m.r. (60 M Hz) τ4.67 (6-H), 4.28 (4-H). The mixture was isomerised with anhydrous oxalic acid, as described in section 3.2.iii.b., to the 4-en-3-one.

d. The Oppenauer oxidation of 17-methyleneandrost-5-en-3β-ol[88]

17-Methyleneandrost-5-en-3β-ol (0.517g) was dissolved in toluene (40ml) and cyclohexanone (7ml) and traces of moisture were removed by distillation of the solvent until clear. A solution of aluminium isopropoxide (1.116g) in toluene (35ml) was added to the gently refluxing steroid solution. The mixture was refluxed for 3 hours, water was added and the organic solvents were removed by steam distillation with a Soxhlet extractor for 1½ hours. The aqueous phase in the reaction flask was extracted twice with chloroform, the combined chloroform extracts washed with dilute hydrochloric acid, saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness. The product, a yellow glass (0.823g), crystallised on standing but smelled strongly of cyclohexanone.

Recrystallisation from acetone gave white crystals of 17-methyleneandrost-4-en-3-one (80mg, 16%); n.m.r. (100 M Hz) τ9.17 (C-18 methyl), 8.80 (C-19 methyl), 5.36 (17=CH_2), 4.27 (4-H).

3.2.iv. 17α,20-Oxido-21-norpregn-4-en-3-one[68][112a]

m-Chloroperbenzoic acid (60% active oxygen, 0.35g, 1.21 mmole active oxygen) was added to a solution of 17-methyleneandrost-4-en-3-one (0.5g, 1.76 mmole) in methylene chloride (20ml). The reaction was allowed to proceed for 2 hours at room temperature. Excess peracid was destroyed with 10% sodium sulphite solution and the reaction mixture was neutralised with saturated sodium bicarbonate solution, washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness. Crystallisation from acetone yielded 17α,20-oxido-21-norpregn-4-en-3-one (0.45g); m.p. 180-184° (lit.,[88] 179-182°); ν_{max} 1675cm^{-1}; n.m.r. (100 M Hz) τ9.14 (C-18 methyl), 8.82 (C-19 methyl), 7.34, 7.26 (d,d,J_{AB} 4 Hz, α-epoxide, C-CH_2-O), 4.28 (4-H); Mass spectrum, M^+, m/e 300.

3.2.v. 17α-Hydroxy-17-hydroxymethylandrost-4-en-3-one[78]

m-Chloroperbenzoic acid (60% active oxygen, 3.9g, 13.6 mmole active oxygen) was added to a solution of 17-methyleneandrost-4-en-3-one (3.86g, 13.6 mmole) in methylene chloride (100ml) and the reaction carried out as described above. T.l.c. showed that the major product of this reaction was more polar than the epoxide and gave a bright blue spot. Recrystallisation from acetone gave 17α-hydroxy-17-hydroxymethylandrost-4-en-3-one (3.1g); m.p. 236° (lit.,[88] 234-236°); n.m.r. (100 M Hz) τ9.01 (C-18 methyl), 8.89 (C-19 methyl), 6.43, 6.38 (d,d,J_{AB} 11 Hz, 17β-CH_2-), 4.25 (4-H).

3.2.vi. 17β-Methylandrost-4-ene-3β,17-diol[80] by reduction of 17α,20-oxido-21-norpregn-4-en-3-one[88]

A solution of 17α,20-oxido-21-norpregn-4-en-3-one (0.45g) in dry ether (25ml) was slowly added to a suspension of lithium aluminium hydride (0.20g) in ether (20ml). The mixture was refluxed for 30 minutes and excess reagent destroyed by careful addition of water. The aqueous layer was separated and washed with ether, the combined ether extracts were washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness. Recrystallisation from acetone gave 17β-methylandrost-4-ene-3β,17-diol (0.36g); m.p. 225-228°; ν_{max} 3410cm^{-1}; n.m.r. (100 M Hz) τ9.31 (C-18 methyl), 8.95 (C-19 methyl), 8.83 (C-20 methyl), 5.85 (m, $w_{\frac{1}{2}}$ 18 Hz, 3α-H), 4.72 (4-H); Mass spectrum, M^+, m/e 304; calculated for $C_{20}H_{32}O_2$ 304.240217, found 304.239655.

3.2.vii. 17-Methylepitestosterone[50]

17β-Methylandrost-4-ene-3β,17-diol (75mg) was dissolved in dry toluene (5ml) and cyclohexanone (1ml). Aluminium isopropoxide (151.2mg) in toluene (5ml) was added to the gently refluxing steroid solution. The reaction mixture was refluxed for 1 hour, allowed to cool and water and concentrated hydrochloric acid were added. The organic layer was separated from the aqueous phase, evaporated to dryness and the residue dissolved in chloroform. The aqueous phase was extracted twice with chloroform and the combined chloroform solutions washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a yellow syrup which smelled strongly of cyclohexanone. Much of the contaminating material

was removed by evaporation in vacuo and by passing a stream of dry nitrogen over the product, at 80°, for 1 hour. Crystallisation from acetone gave long needles of 17-methylepitestosterone (30mg); m.p. 167-168° (lit.,[111b] 182°); t.l.c., one spot, less polar than the starting material; Mass spectrum, M^+, m/e 302.

3.2.viii. 17β-Methylandrost-4-ene-3β,17-diol[80] by reduction of 17-methylepitestosterone[118]

A solution of 17-methylepitestosterone (15mg) in methanol (0.6ml) and ethyl acetate (0.15ml) was mixed with a solution of sodium borohydride (5mg) in methanol (0.6ml) and ethyl acetate (0.15ml) and kept at room temperature for 24 hours. Glacial acetic acid (0.2ml) was added followed by dilute hydrochloric acid. The steroid was extracted into ether and the ether solution washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a white crystalline product (10.6mg); n.m.r. (100 M Hz) τ9.31 (C-18 methyl), 8.94 (C-19 methyl), 8.82 (C-20 methyl), 5.86 (m, $w_{\frac{1}{2}}$ 18 Hz, 3α-H), 4.72 (4-H). A small peak at τ9.01 (C-19 methyl) indicated that the product contained a small proportion of 17β-methylandrost-4-ene-3α,17-diol. Recrystallisation from acetone gave 17β-methylandrost-4-ene-3β,17-diol (2.5mg); m.p. 226-228°; $[\alpha]_D$ + 8° (C = 0.18).

3.2.ix. 17α-Methylandrost-4-ene-3β,17-diol[118]

A solution of methyltestosterone (0.41g) in methanol (16.4ml) and ethyl acetate (4.1ml) was mixed with a solution of sodium borohydride (0.106g) in methanol (16.4ml) and ethyl

acetate (4.1ml) and the reaction carried out as described above. The white product (0.443g) was sparingly soluble in ether and crystallisation from acetone gave 17α-methylandrost-4-ene-3β,17-diol (0.199g); m.p. 167-169° (lit.,[118] 166-170°); n.m.r. (100 M Hz) τ9.13 (C-18 methyl), 8.94 (C-19 methyl), 8.81 (C-20 methyl), 5.86 (m, $w_{\frac{1}{2}}$ 14 Hz, 3α-H), 4.72 (4-H); t.l.c., similar polarity to 17β-methylandrost-4-ene-3β,17-diol; $[\alpha]_D$ + 14° (C=0.25); Mass spectrum, M^+, m/e 304.

3.2.x. 17-Epimethandrostenolone[81][119]

A solution of 17β-methylandrost-4-ene-3β,17-diol (0.35g, 1.15 mmole) and DDQ (0.57g, 2.5 mmole) in dry dioxane (20ml) was refluxed for 6 hours. The solvent was removed by evaporation in vacuo and the residue dissolved in benzene and filtered through a layer of alumina (10g). Elution with benzene removed three low polarity components and then a mixture of two components was eluted with benzene-chloroform (1:1); the latter mixture was retained. Rechromatography of this fraction achieved partial separation and each compound was isolated by crystallisation from the fraction in which it was most abundant. The less polar compound (40mg) was identified as 17-methylepitestosterone; m.p. 180-182° (lit.,[111b] 182°); ν_{max} 3410,1675cm^{-1}; n.m.r. (100 M Hz) τ9.28 (C-18 methyl), 8.81 (C-20 methyl), 8.81 (C-19 methyl), 4.28 (4-H) and the second compound (140mg) as 17-epimethanødrostenolone; m.p. 221°; ν_{max} 3410,1665,1636,910cm^{-1}; n.m.r. (100 M Hz) τ9.26 (C-18 methyl), 8.82 (C-20 methyl), 8.77 (C-19 methyl), 3.92 (d, 2Hz, 4-H), 3.78 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.94 (d, 10 Hz, 1-H); $[\alpha]_D$ + 3° (C=0.50); Mass spectrum, M^+, m/e 300; calculated for $C_{20}H_{28}O_2$ 300.208919, found 300.210430.

3.3. The comparison of methandrostenolone and 17-epimethandrostenolone

Adhikary reported[32] that on the microscale methandrostenolone was only distinguishable from its less polar metabolite (M) by a very small difference in retention times on g.l.c. [see table 1, page 23]. Consequently methandrostenolone and its 17-epimer were compared prior to comparison of 17-epimethandrostenolone and M.

3.3.i. Thin layer chromatography

Methandrostenolone and its C-17 epimer were not separated by the solvent systems, benzene-ethanol (9:1) and chloroform-methanol (9:1). Because of the presence of the $\Delta^{1,4}$-dien-3-one chromophore both compounds were visible when the plates were viewed under ultraviolet light. Development with sulphuric acid produced identical orange-brown spots after heating.

3.3.ii. Infrared spectroscopy

The infrared spectra of both epimers of methandrostenolone were very similar and only minor differences could be discerned in the fingerprint region. Absorptions at ν_{max}1665 and 1630cm^{-1}, characteristic of the $\Delta^{1,4}$-dien-3-one system were present in the spectra of both compounds.

Ananchenko *et al.*[89] were unable to distinguish between the C-17a epimeric 17a-alkyl-3-methoxy-D-homooestra-1,3,5(10),8-tetraen-17a-ols by infrared spectroscopy but noted small differences in the spectra of C-17a epimeric 17a-alkyl-19-nor-D-homotestosterones.

3.3.iii. Melting points

The melting point of 17-epimethandrostenolone (221°) differed considerably from that of methandrostenolone (163°). Other pairs of C-17 epimers which have been found to show such a difference, the 17α-alcohol having the higher melting point in all cases are testosterone (155°)[111b] and 17-epi-testosterone (221°),[111b] methyltestosterone (164°)[111b] and 17-methylepitestosterone (182°)[111b] and 17α-methylandrost-4-ene-3β,17-diol (166-169°) and 17β-methylandrost-4-ene-3β,17-diol (225-228°).

3.3.iv. Optical Rotations

Until approximately twenty years ago there was confusion about the C-17 configuration of natural steroids such as testosterone, which was originally assigned the 17α-hydroxyl configuration.[92,111c] When the true structures of such compounds were determined it was found that inversion from the 17β- to the 17α-configuration was accompanied by a laevorotatory shift of the optical rotations of the compounds.[111c]

Optical Rotations	$[\alpha]_D^{\beta\text{-ol}}$	$[\alpha]_D^{\alpha\text{-ol}}$	$[\alpha]_D^{\alpha}-[\alpha]_D^{\beta}$
Testosterones[111b]	+109	+71.5	-37.5
Methyltestosterones[111b]	+76	+67	-9
Methandrostenolones	+20	+3	-17
17-Methylandrost-4-ene-3β,17-diols	+14	+8	-6

Table 2. The optical rotation of pairs of C-17 epimeric steroidal alcohols

The optical rotations of the C-17 epimers of methandrostenolone and 17-methylandrost-4-ene-3β,17-diol showed differences typical of the structures of the compounds [table 2].

3.3.v. Nuclear magnetic resonance spectroscopy

The resonances of the three A-ring protons were identical in the spectra of both epimers of methandrostenolone, τ3.92 (d, 2 Hz, 4-H), 3.78 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.94 (d, 10 Hz, 1-H), as expected. However comparison of the methyl resonances of the three pairs of epimeric 17-hydroxy-17-methyl compounds synthesised [table 3] showed that the chemical shift of the C-18 methyl group was characteristic of the configuration at C-17. Indeed

	C-18 methyl		C-19 methyl		C-20 methyl	
configuration at C-17	α-OH	β-OH	α-OH	β-OH	α-OH	β-OH
Methandrostenolones	9.26	9.07	8.77	8.77	8.81	8.82
Methyltestosterones	9.28	9.09	8.81	8.79	8.81	8.79
17-Methylandrost-4-ene-3β,17-diols	9.31	9.13	8.95	8.94	8.83	8.81

Table 3. The chemical shifts (τ values) of the C-18, C-19 and C-20 methyl groups of pairs of 17-epimeric steroidal alcohols.

Ananchenko et al.[89] have used the chemical shift of the C-18 methyl group to assign the configuration at C-17a in substituted D-homooestrenes and D-homo-19-nortestosterones. Their interpretation was based upon unpublished work on the spectra of cyclic carbinols by Berezin and Musher, who found that the resonance line of a methyl group was shifted to

weaker field the closer the protons were to the oxygen of a hydroxyl group.

3.3.vi. Gas-liquid chromatography

Methandrostenolone and 17-epimethandrostenolone were chromatographed separately and as mixtures on OV-1 and QF-1 columns at several temperatures between 225 and 250°C. The latter epimer had a fractionally shorter retention time (0.978 of the R_t of methandrostenolone) when the compounds were chromatographed separately, but a mixture could not be resolved on OV-1. Slight separation of the heads of the peaks of each component was obtained on QF-1 at 240°, but this was only possible for an approximately equimolar mixture of the epimers. Hexamethyldisilazane was injected onto the QF-1 column in an attempt to reduce tailing and thus improve separation but the chromatography was not improved. The epimeric 17-methytestosterones could not be resolved on an OV-1 column.

The use of trimethylsilylation has resulted in the successful g.l.c. of many steroids which would otherwise have been unsuitable because of poor volatility and instability at the temperatures necessary for chromatography. Replacement of active hydrogen atoms by the trimethylsilyl ether function leads to decreased polarity and increased volatility because of reduced intermolecular hydrogen bonding.[121a] Since the introduction of the trimethylsilyl group may accentuate minor differences between closely related compounds,[121b] silylation of the epimers of methandrostenolone was attempted.

A method employing hexamethyldisilazane and dimethylchlorosilane at room temperature, which has been used success-

fully for the preparation of the trimethylsilyl ethers of corticosteroids[122] was examined. The products of this reaction should be particularly suitable for direct injection into a g.l.c. instrument or mass spectrometer because of the absence of acetamide derivatives which lead to high backgrounds.[122] However, silylation of the tertiary C-17 hydroxyl group was not achieved under these conditions.

In 1970 Brooks et al.[25] reported details of the 17β-trimethylsilyl ethers of twelve steroid drugs, including methandrostenolone, which contained 17α-methyl,-ethyl,-propyl, -allyl and -ethynyl substituents. These derivatives were prepared by the method of Makita and Wells,[123] who used hexamethyldisilazane and trimethylchlorosilane as the silylating reagents. Methandrostenolone and 17-epimethandrostenolone were successfully silylated by this method, although g.l.c. showed that the reaction did not go to completion. The derivatives of the epimers were well resolved and the peak shapes were also improved by the absence of tailing. When chromatographed on OV-1 at 235° the retention times of the derivatives compared to those of the free steroids were 1.077 ± 0.014 (9 runs) for methandrostenolone and 0.791 ± 0.015 (7 runs) for 17-epimethandrostenolone. Consequently the C-17 epimers of methandrostenolone were easily differentiated by g.l.c. of their trimethylsilyl ethers.

In later experiments the methods of Chambaz and Horning[124] and Sakauchi and Horning,[125] developed for the silylation of compounds containing highly hindered hydroxyl groups, were investigated. Trimethylsilylimidazole and tri-

methylchlorosilane were employed in the former method and in combination with bistrimethylsilylacetamide in the latter, and the reaction mixtures were heated in both cases. T.l.c. showed that under these conditions the only products of the silylations of the epimers of methandrostenolone were the low polarity derivatives, uncontaminated by free steroid. This was verified by g.l.c.

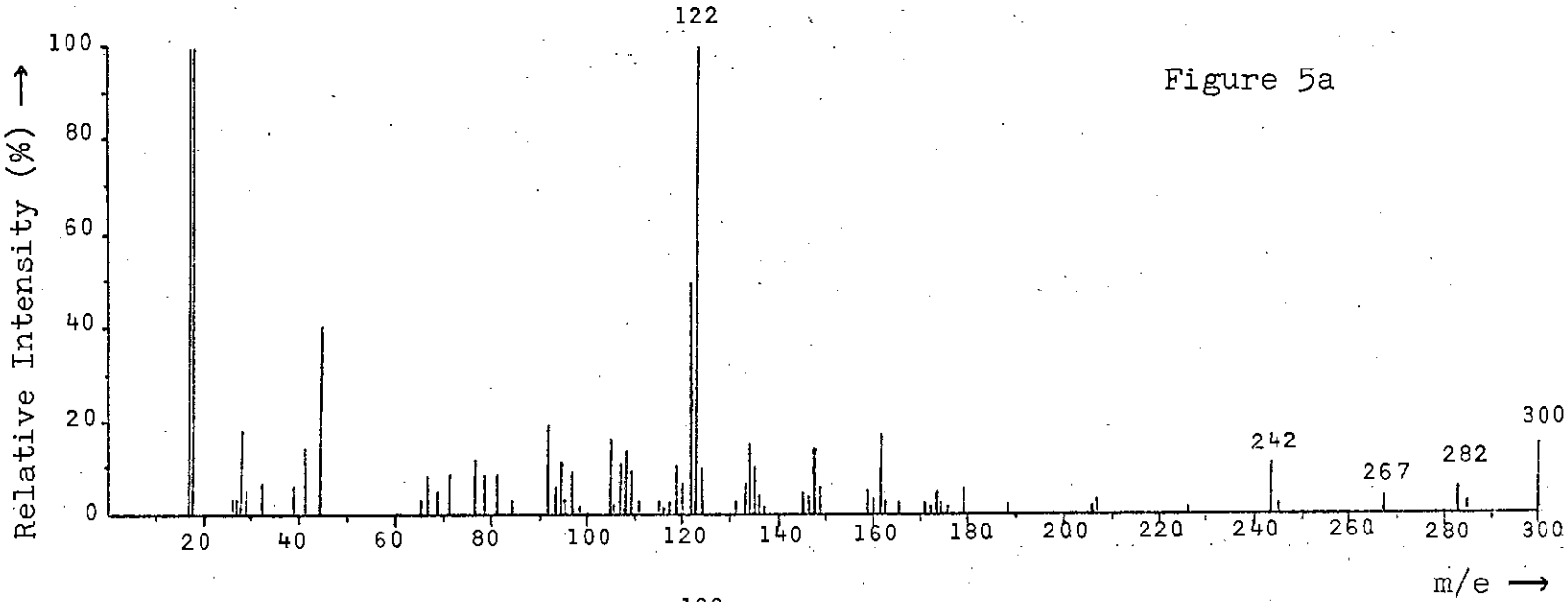
3.3.vii. Mass spectrometry

The use of mass spectrometry in biological, pharmacological and medical investigations has increased rapidly in recent years because of the very low sample requirements of the technique.[126a] When mass spectrometry was first used for the study of steroids it was thought that compounds differing only in their stereochemistry at one position, such as epimeric hydroxy steroids,[127] would be indistinguishable.[128] However, the refinement of techniques, particularly for the introduction of samples into the spectrometer, has enabled many epimeric pairs of compounds to be differentiated.[128] All-glass inlet systems and direct introduction of the sample into the ion source result in diminished thermal reactions and thus in ions characteristic of electron-induced fragmentation.

The problem of the differentiation of epimeric alcohols by mass spectrometry was examined by Biemann and Seibl[129] who observed that the spectra of epimeric cyclic alcohols, including steroidal alcohols, were generally very similar but that there were distinct differences in the abundances of the molecular ion (M^+) and the ion arising from loss of

water (M-18). In the spectra of secondary alcohols these authors found that M^+ was more intense (and M-18 less intense) for the equatorial alcohol, which was less crowded and therefore more stable than M^+ of the axial alcohol. The converse was true for the spectra of tertiary alcohols, in which the axial alcohol gave the most intense M^+. This was interpreted as an effect of the crowding produced by the equatorial alkyl group which was larger than the hydroxyl group.

The mass spectrometry of epimeric steroidal alcohols has been extensively studied by a group of Russian workers.[89, 130-133] In an investigation[89] of the C-17a configuration of 17a-alkyl-D-homooestra-1,3,5(10),8-tetraen-17a-ols and 17a-alkyl-19-nor-D-homotestosterones they compared the ratios of the intensities of the M-18 and M^+ ions and found that this ratio was much greater for one compound in each pair than for the other. Applying the "crowding" concept of Biemann and Seibl[129] they concluded that the ratio was greatest for those compounds in which the 17a-alkyl group was β (equatorial) and thus closest to the C-18 methyl group. This assignment of configuration was confirmed by comparison with assignments made on the basis of chemical reactivity and n.m.r. spectroscopy. Examination[132] of the spectra of 17-alkyloestradiols and 17-alkyl-19-nortestosterones showed that the difference in the ratio of M-18 to M^+ for pairs of epimers was much less pronounced than in the D-homo series, but that dehydration of the 17α-hydroxy compound still proceeded more readily than that of the 17β-epimer. It has, however, been reported[132] that no differences between



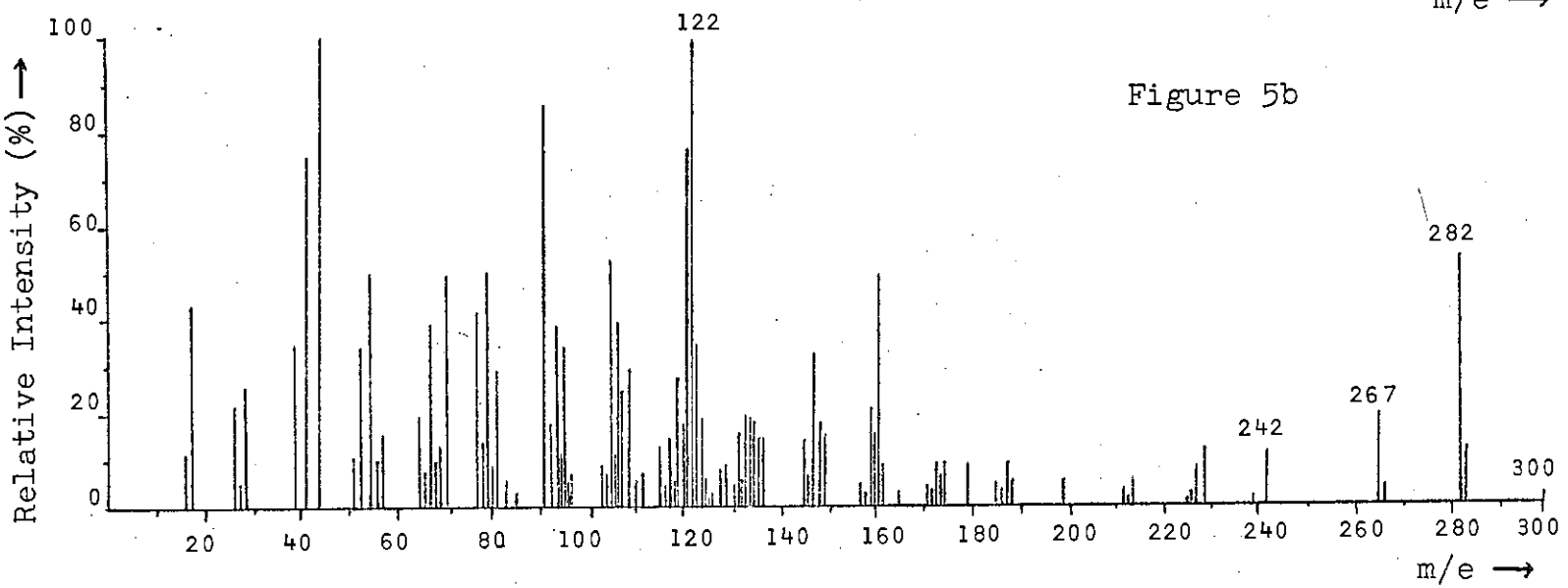
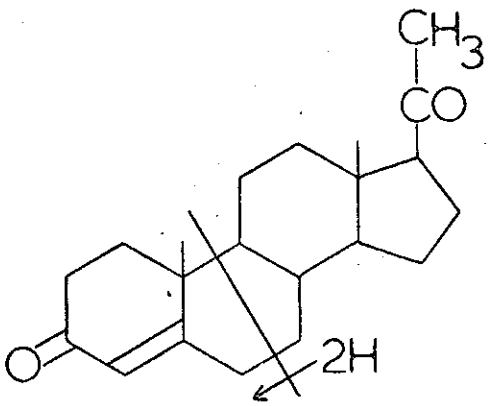


Figure 5. The mass spectra of a) methandrostenolone and b) 17-epimethandrostenolone at 125°C and 120°C, respectively.

the mass spectra of the 17a-epimers of 17a-methyl-D-homotestosterone or between those of 17a-ethyl-D-homotestosterone could be detected. Comparison of the mass spectra of methyltestosterone and its C-17 epimer [diagram 9] showed no significant difference in the intensities of the M-18 and M^+ ions.

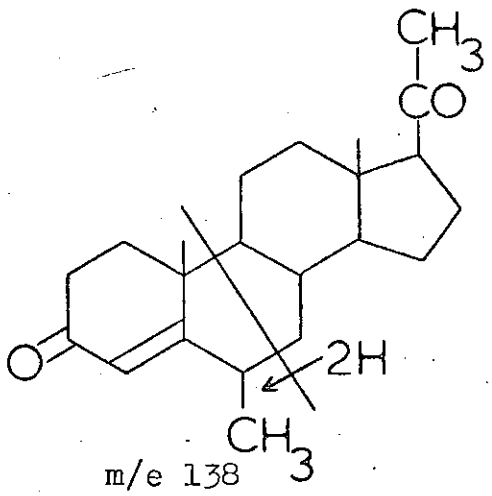
Differences between the mass spectra of epimeric secondary alcohols in the progesterone series were studied by Zaretskii *et al.*[131] who concluded that the ratio $M-18/M^+$ would be greater than unity for those alcohols in which the hydroxyl group was axial and less than unity for equatorial alcohols. Grostic and Rinehart[134] obtained similar results for the particular steroids (11α- and 11β-hydroxyprogesterone) studied by the Russian workers[131] but found that other hydroxy-substituted progesterones had more intense molecular ions than dehydration ions, regardless of the configuration of the hydroxyl group. These results[134] illustrate the necessity for standard compounds of known configuration, since it had been suggested[131] that the ratio $M-18/M^+$ could be considered diagnostic of configuration if a known compound was not available for comparison.

Comparison of the mass spectra of methandrostenolone [figure 5a] and 17-epimethandrostenolone [figure 5b] showed that M^+ was greater than M-18 for the former compound and less than M-18 for the latter. Thus the "crowding" effect[129] between the 17β-methyl and C-18 methyl groups of 17-epimethandrostenolone operated to destabilise the molecular ion [figure 6]. Spectra were obtained at a variety of temperatures and it was found that the ratio $M-18/M^+$ was always less



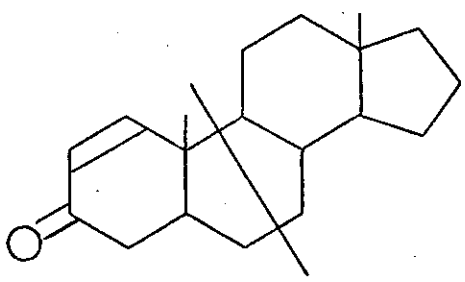
m/e 124

[82]



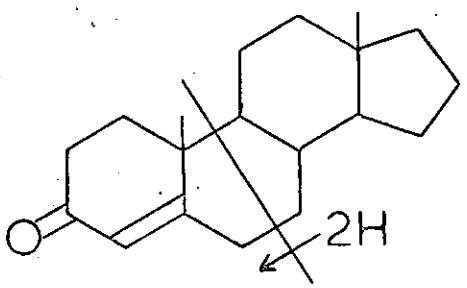
m/e 138

[83]



m/e 122

[84]



m/e 124

[85]

than unity for methandrostenolone and greater than unity for its 17-epimer [table 4]. The fact that this relationship was true over a range of temperatures was important for application to metabolic samples since the presence of contaminants could alter the temperature corresponding to maximum ion pressure.

The high mass region of the spectrum is particularly useful for information concerning the nature of substituents.[135] In the spectra of methandrostenolone and its 17-epimer the ions in this region were generally of low intensity in comparison to the base peak at m/e 122. Peterson,[136] in a study of the mass spectra of a variety of highly substituted pregnanes and pregnenes, observed that the base peaks of progesterones [82] and 6-methylprogesterones [83] were at m/e 124 and m/e 138 respectively. He concluded that these ions arose from fragmentation across the 6-7 and 9-10 bonds with transfer of two hydrogen atoms to the charged A-ring fragment. The mass spectra of androst-1-en-3-ones[84] and androst-4-en-3-ones [85] were studied by Shapiro and Djerassi.[137] These compounds give rise to very intense ions, generally the base peaks, at m/e 122[86] and m/e 124 [87], respectively.

Experiments with compounds appropriately labelled with deuterium have established that the m/e 122[86] and m/e 124 [87] ions in the spectra of Δ^1-en-3-ones and Δ^4-en-3-ones, respectively, arise by related processes[137,138] [diagram 10]. The 9-10 bond is first cleaved and migration of the 8β-hydrogen atom to C-10 occurs with formation of an 8-9 double bond[88]. In the Δ^1-enone series the 5α-hydrogen atom migrates to C-7 to give the A-ring fragment, m/e 122[86].

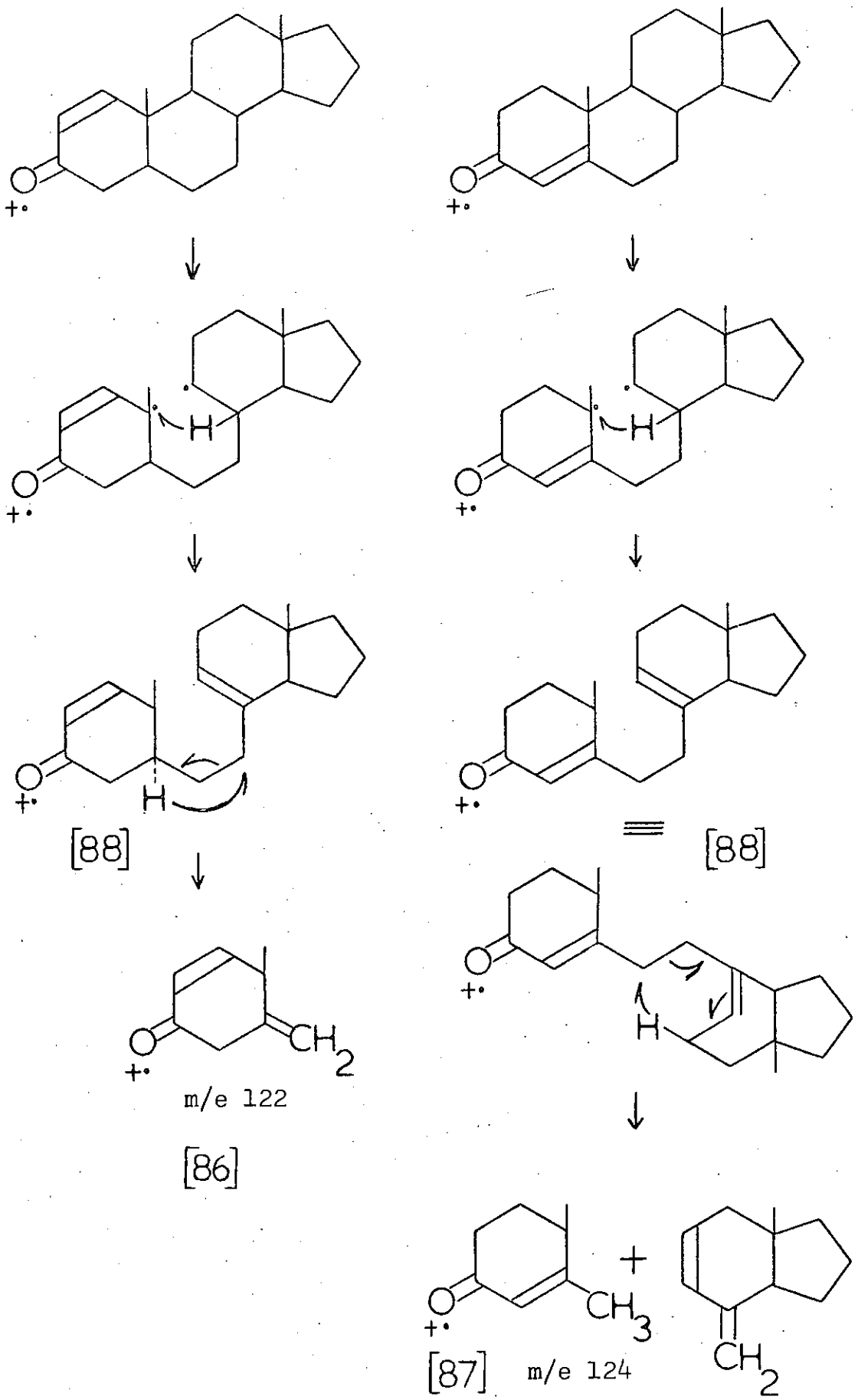


Diagram 10. Mass spectral fragmentation processes of androst-1-en-3-one and androst-4-en-3-one.

Intensities (% base peak, m/e 122)	m/e300 M^+	m/e282 M-18	m/e267 M-18-15	m/e242 M-58	m/e282 / m/e300	m/e267 / m/e300	m/e242 / m/e267	Temp. (°C)
Methandrostenolone	15.6	6.4	3.7	11.0	0.41	0.27	3.0	125
	27.1	7.2	4.2	13.9	0.27	0.16	3.3	90
	24.2	6.9	5.2	19.0	0.29	0.22	3.7	90
Mean ratios, methandrostenolone					0.32	0.21	3.3	
17-Epimethandrostenolone	1.1	62.2	11.1	2.2	56.5	10.1	0.20	150
	1.5	94.2	17.7	4.4	62.8	11.8	0.25	160
	0.9	71.0	14.3	4.2	78.9	15.9	0.29	120
Mean ratios, 17-epimethandrostenolone					66.1	12.6	0.25	

Table 4. The intensities (% base peak) of four characteristic ions in the spectra of methandrostenolone and its 17-epimer.

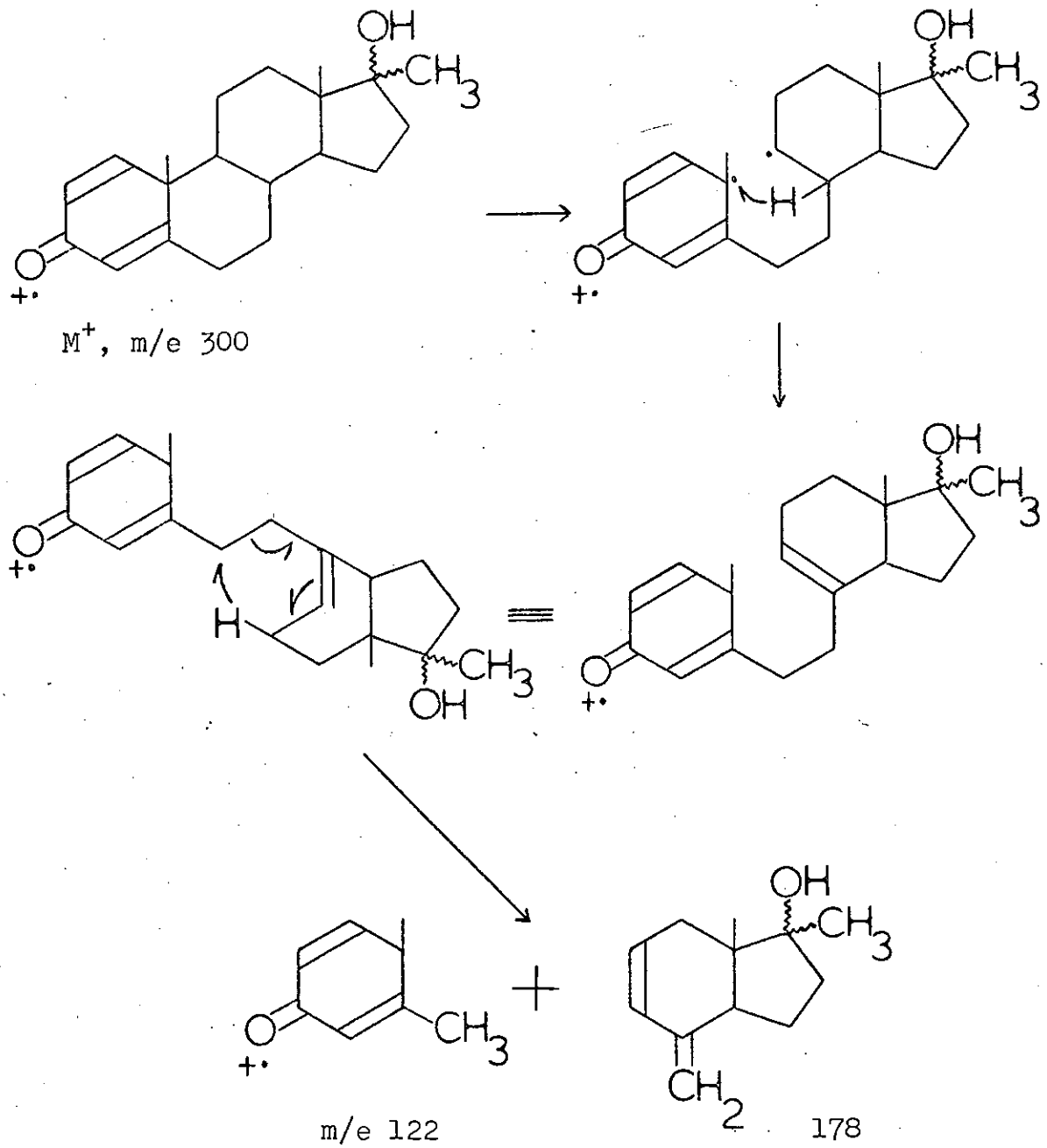


Diagram 11. Mass spectral fragmentation of methandrostenolone and its 17-epimer.

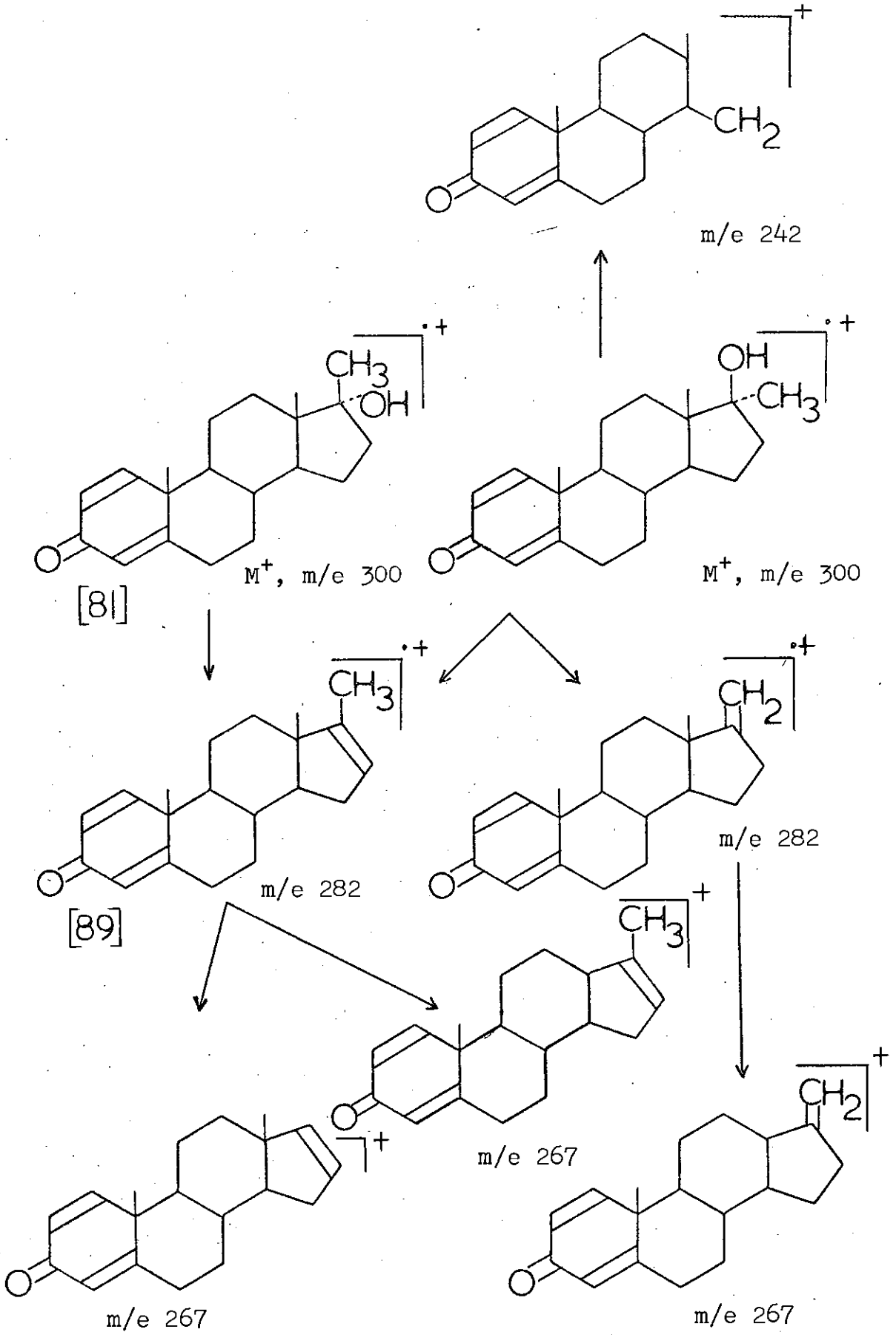


Diagram 12. Mass spectral fragmentations of methandrostenolone and its 17-epimer.

This migration cannot occur in the Δ^4-enone series and thus rotation of the 7-8 bond occurs such that a C-11 hydrogen atom migrates to C-6 with formation of the ion, m/e 124[87]. Consequently, the base peak (m/e 122) in the spectra of methandrostenolone and its 17-epimer must arise by the same mechanism [diagram 11] as the m/e 124 ion in the spectra of Δ^4-en-3-ones.

Zaretskii et al.[130] studied the mass spectra of the epimeric 3-methoxy-17a-trideuteromethyl-D-homooestra-1,3,5(10),8-tetraen-17a-ols and found that dehydration of the 17aα-alcohol gave the endocyclic Δ^{16}-double bond whereas dehydration of the 17aβ-alcohol occurred less readily and gave a mixture of the endo- and exocyclic double bonds. The M-18-15 ion in the spectrum of the α-alcohol was shown to arise from elimination of either the C-17 or C-18 methyl groups. Consequently, the intensity of this peak was greater than that of the corresponding peak in the spectrum of the β-alcohol, which was diminished because of formation of the 17-methylene M-18 ion.

As expected, the ratio M-18-15/M^+ was greater for 17-epimethandrostenolone than for methandrostenolone [table 4], showing that endocyclic elimination of water [89] was predominant in the former molecule [81][diagram 12]. Elimination of the C-19 methyl group presumably contributed to the M-18-15 ion in the epimers of methandrostenolone, since Popov et al.[139] have shown, by deuterium labelling, that the C-19 methyl group is eliminated from 5α-androstan-17-one three times as readily as the C-18 methyl group.

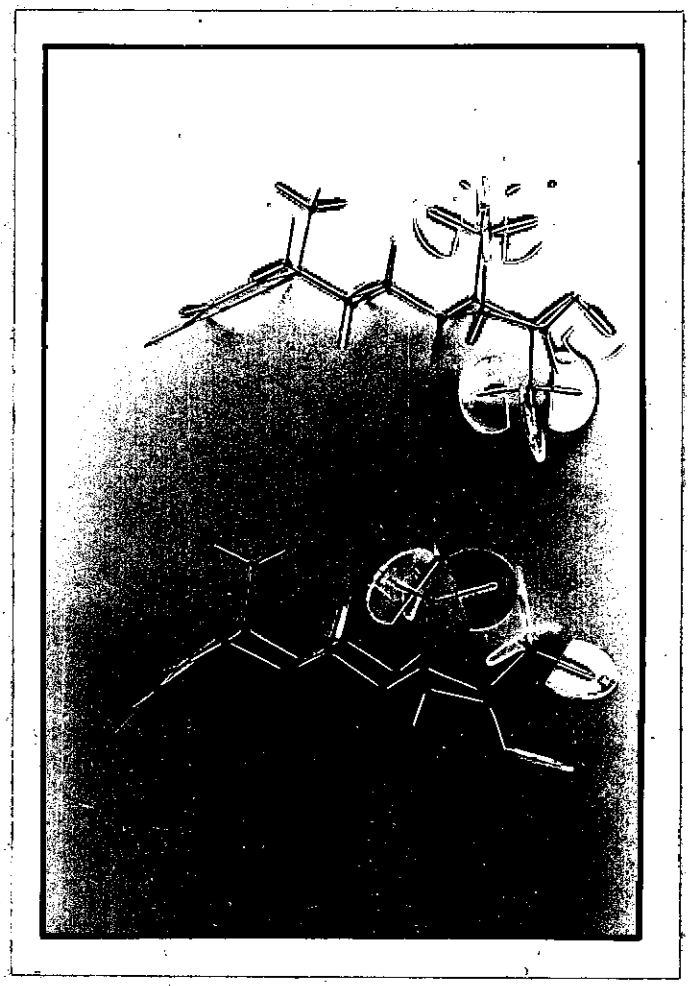


Figure 6. Dreiding models of methandrostenolone and its 17-epimer showing the crowding around the 13-17 bond in the latter compound.

Fragmentation of the D-ring of C-17 alcohols is well known[89,108,132,140] and Ananchenko et al.[89] observed that cleavage of the 13-17a bond in 17a-alkyl-3-methoxy-D-homooestra-1,3,5(10),8-tetraen-17a-ols occurred most readily for the less crowded β-hydroxy epimers. The ion arising from fragmentation across the 13-17 and 15-16 bonds (m/e 242, M-58) was significant in the spectrum of methandrostenolone[25] but of very low intensity in that of the 17α-hydroxy epimer [see figure 6]. It thus appears that methandrostenolone and 17-epimethandrostenolone fragment preferentially by different mechanisms, D-ring cleavage being more favourable for the former and dehydration and methyl elimination for the latter. Comparison of the ratio M-58/M-18-15 for the epimers showed that it was greater than unity for methandrostenolone and less than unity for 17-epimethandrostenolone [table 4]. Fragmentation across the 13-17 and 14-15 bonds to give a very low intensity ion at m/e 227 occurred in both epimers.

Spectra of methandrostenolone contained a low intensity metastable ion at m/e 265.2 associated with the fragmentation, M^+ to M-18. Those of 17-epimethandrostenolone contained a very low intensity metastable ion at m/e 252.7, corresponding to the transition, M-18 to M-18-15. Neither compound gave both metastable ions.

Although the molecular ions of most trimethylsilylated compounds are very weak[121c] the mass spectra of these derivatives have proved useful for the detection of certain functional groups, such as the Δ^5-en-3β-ol system, the silyl ethers of which give rise[121d] to intense ions at m/e 129 and

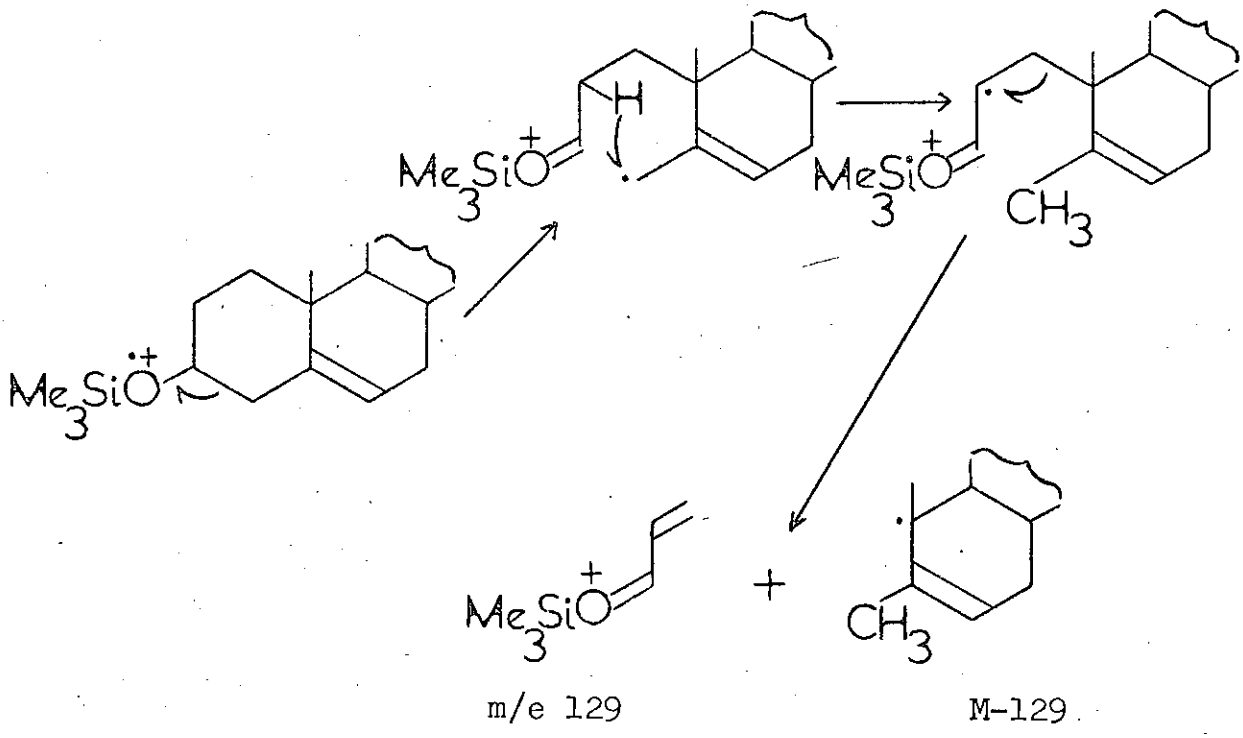
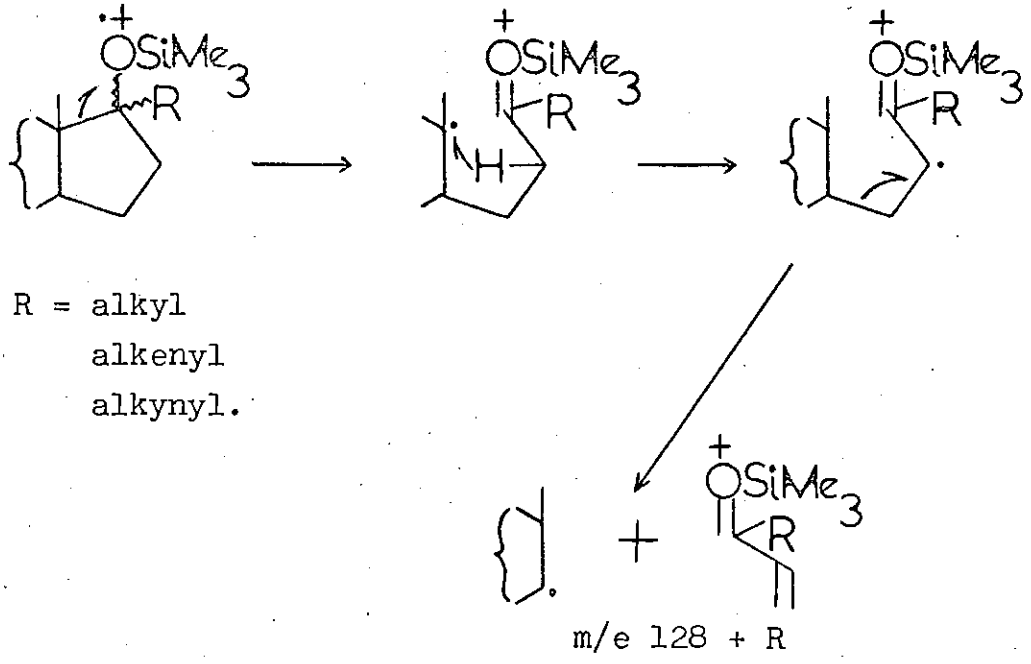


Diagram 13. Mass spectral fragmentation of Δ^5-en-3β-trimethylsilyl ethers.



R = alkyl
alkenyl
alkynyl.

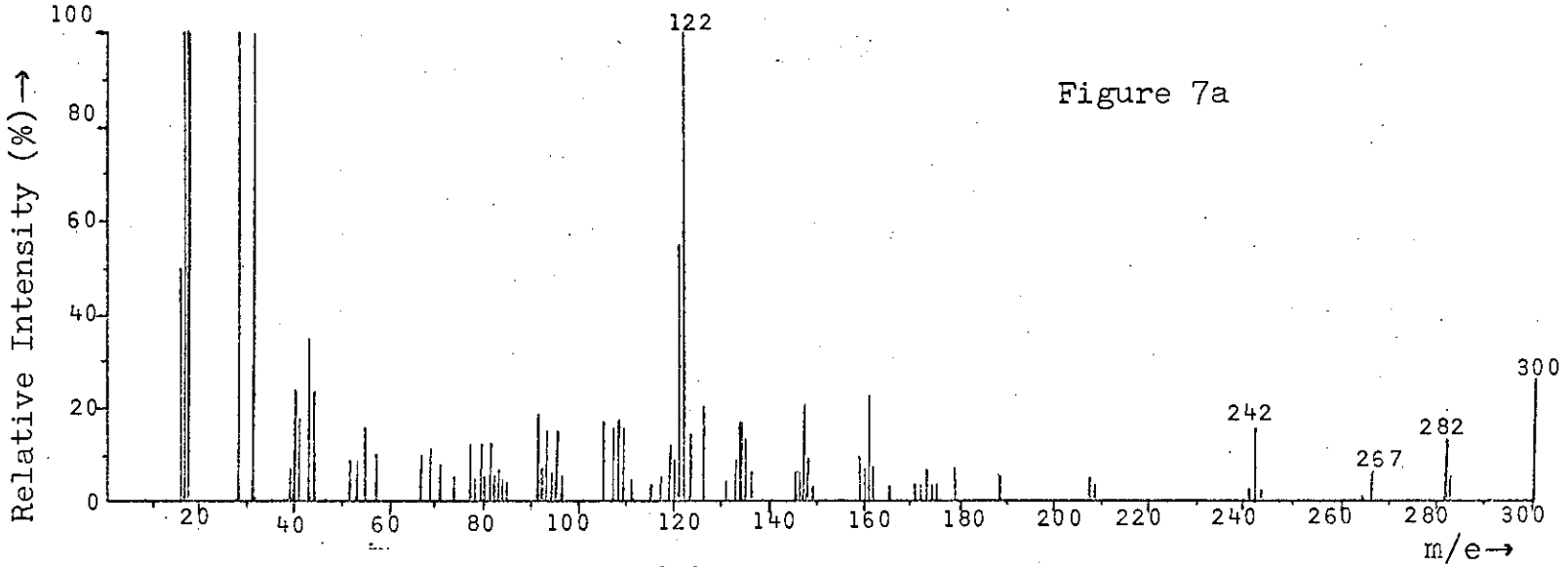
Diagram 14. Mass spectral fragmentation of 17-substituted-17-trimethylsilyl ethers.

m/e M-129. By the use of deuterium labelling Diekman and Djerassi[141] have shown that the m/e 129 fragment contains carbons 1, 2 and 3 of the A-ring with the trimethylsilyl ether function [diagram 13].

Brooks et al.[25] found that the mass spectra of the trimethylsilyl ethers of steroid drugs, including methandrostenolone, contained abundant ions at m/e 128 + R, characteristic of a tertiary 17-hydroxy steroid where R = alkyl, alkenyl and alkynyl [diagram 14]. The spectra of the trimethylsilyl ethers of methandrostenolone and 17-epimethandrostenolone were very similar, the base peak of both being at m/e 143 (128 + 15). The molecular ion (m/e 372) was of low intensity in both spectra and the only other ions of importance were those at m/e 73 [$(CH_3)_3Si^+$], m/e 75 [$\overset{+}{O}H = Si(CH_3)_2$] and m/e 282[M-$(CH_3)_3SiOH$]. The influence of the trimethylsilyl group on the direction of fragmentation was obvious from the almost complete loss of m/e 122, the base peak of the free steroids.

3.3.viii. Gas chromatography-mass spectrometry

The stability of methandrostenolone and its 17-epimer during g.l.c. was examined by preparative chromatography followed by mass spectrometry of the condensed effluent. A sample of methandrostenolone was chromatographed and the effluent collected as the peak was recorded. The condensate was eluted, rechromatographed and the effluent again collected, eluted and then subjected to mass spectrometry. The retention times were the same for both chromatograms and the mass spectrum [figure 7a] was similar to that of authentic



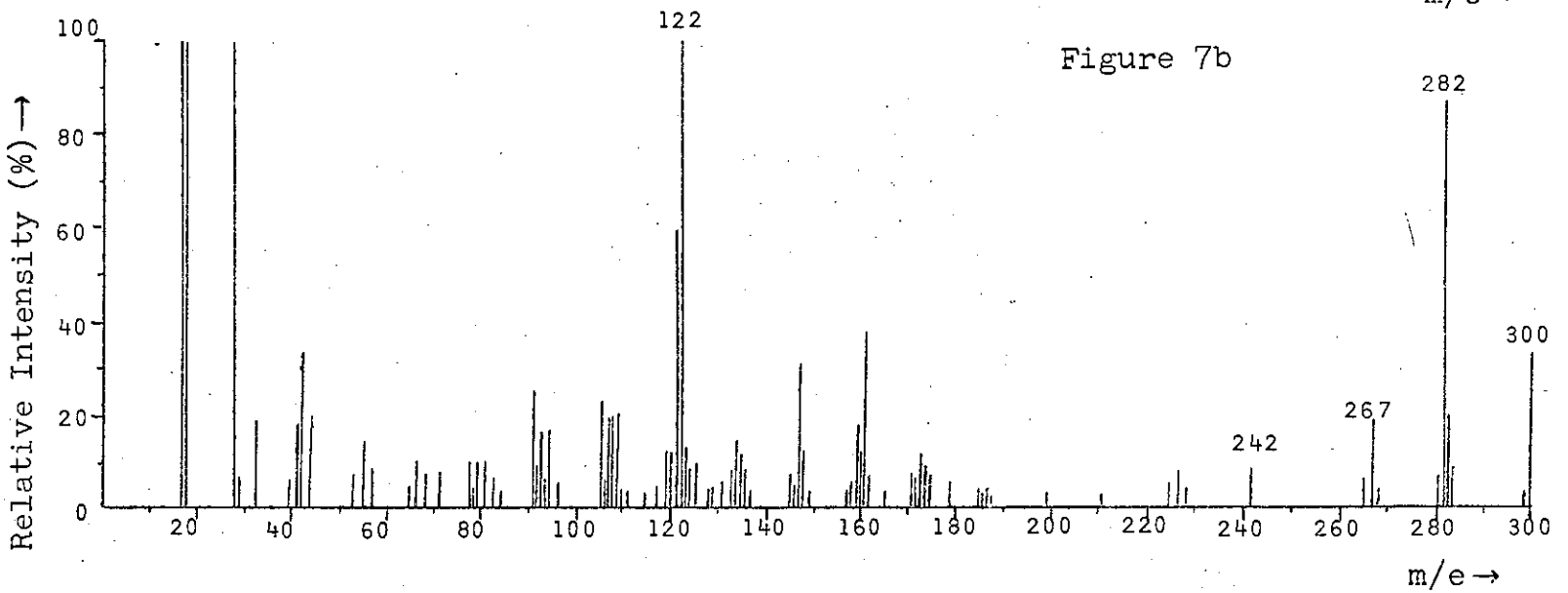


Figure 7. The mass spectra of a) methandrostenolone at 80°C and b) its 17-epimer at 95° after preparative g.l.c.

methandrostenolone [figure 5a]. 17-Epimethandrostenolone was examined in the same way and again the rechromatographed material had the same retention time as the peak from which it was collected. The mass spectrum of the twice-chromatographed 17-epimethandrostenolone [figure 7b] was similar to that of the pure compound [figure 5b].

Table 5 shows the ratios of the intensities of the characteristic ions from the spectra of methandrostenolone and 17-epimethandrostenolone after preparative g.l.c. The mean values of the intensity ratios from spectra of methandrostenolone obtained at 80 and 90°C were similar to those obtained from spectra of the unchromatographed compound [table 4, page 59]. Spectra of 17-epimethandrostenolone were taken over a wide range of temperatures (60-150°C) and although the intensity ratios did not differ so greatly from those of methandrostenolone as did those of the unchromatographed compound [table 4] the spectra of the two epimers were still significantly different.

The reason for the diminished m/e 282/m/e 300 and m/e 267/m/e 300 ratios, corresponding to an apparent increase in the stability of the molecular ion of 17-epimethandrostenolone after preparative g.l.c. is uncertain. However, a temperature effect in the mass spectrometer may have been responsible since the g.l.c. retention time and the mass spectra, which contain no ions other than those of 17-epimethandrostenolone, do not suggest degradation of the steroid on the column.

Combined gas chromatography-mass spectrometry (g.c.-m.s.) has been used extensively for the identification of

Intensities (% base peak, m/e 122)	m/e300 M^+	m/e282 M-18	m/e267 M-18-15	m/e242 M-58	m/e282 / m/e300	m/e267 / m/e300	m/e242 / m/e267	Temp. (°C)
Methandrostenolone	27.8	13.0	7.0	15.7	0.47	0.25	2.2	80
	30.4	13.1	4.4	13.9	0.43	0.14	3.0	90
Mean ratios, Methandrostenolone					0.45	0.20	2.6	
17-epimethandrostenolone	9.5	80.9	16.7	7.1	8.5	1.8	0.48	60
	18.9	100.0	22.2	8.9	5.3	1.2	0.40	80
	33.3	86.7	19.4	8.3	2.6	0.58	0.43	90-100
	54.8	83.5	21.9	11.0	1.5	0.40	0.50	110
	77.3	72.7	27.3	13.6	0.94	0.29	0.50	150
Mean ratios, 17-epimethandrostenolone					3.8	0.85	0.46	

Table 5. The intensities (% base peak) of four characteristic ions in the spectra of methandrostenolone and its 17-epimer after preparative g.l.c.

mammalian steroids from plasma, urine and faeces.[126b] Since it had been demonstrated that methandrostenolone and its 17-epimer gave their characteristic, distinguishable mass spectra after g.l.c. the metabolism of methandrostenolone appeared to be ideally suited to examination by g.c.-m.s.

A Pye 104 gas chromatograph linked to an MS-20 spectrometer was used to obtain spectra from methandrostenolone and 17-epimethandrostenolone. In order to protect the membrane separator the oven temperature was restricted by a thermal cut-out at 240° and losses may have occurred through condensation in the transfer line which was operated at 200°C. However, the apparatus was particularly unsatisfactory for the analysis of steroids because of its poor resolution at m/e values greater than 200. Consequently, it was impossible to count the spectrum to the molecular ion because of the background noise from column bleed combined with the low intensities of the high mass ions. A computer link-up was not available for analysis of the spectra and thus the metabolism of methandrostenolone could not be examined by g.c.-m.s.

The technique of single-ion monitoring can be used to locate those compounds which are known to give rise to a characteristic fragment. Lawson and Brooks[27] have examined the metabolites of methandrostenolone by g.c.-m.s. of the trimethylsilylated urine extracts, with monitoring of the m/e 143 ion. By this method they have reported the detection of nanogram amounts of 6β-hydroxymethandrostenolone in the free fraction and tentatively, a compound with one additional

double bond, one with a reduced double bond, two isomers of 6β-hydroxymethandrostenolone and an isomer of methandrostenolone. In the conjugated fraction they found two major metabolites, one which appeared to contain a 6-hydroxy-Δ^4-en-3-one system and the other a 3-hydroxy analogue of this.

3.4. Experimental section. The synthesis of trimethylsilyl ethers.

3.4.i.[122] A mixture of equal volumes of a 40% solution of hexamethyldisilazane in benzene and a 10% solution of dichlorodimethylsilane in benzene was added to the solid steroid and the reaction allowed to proceed overnight at room temperature. The solvent was removed by evaporation in vacuo and the residue extracted with n-hexane. The precipitated silicon compounds were removed by filtration and the solvent evaporated to give a suitable concentration of steroid, which was injected directly into the g.l.c. instrument.

Tertiary alcohols, such as methandrostenolone and its 17-epimer were not silylated by this method.

3.4.ii.[25,123] Hexamethyldisilazane (10 drops) and trimethylchlorosilane (1 drop) were added to a solution of the steroid (1mg) in dry pyridine (4 drops). The mixture was left at room temperature overnight and then the solvents were removed by evaporation with a stream of nitrogen. Ethyl acetate was added to the residue and the solid byproducts removed by filtration.

T.l.c. of the filtrates after silylation of methandrostenolone and 17-epimethandrostenolone showed two similar components in each sample, the major of very low polarity and the other corresponding to the free steroid. G.l.c. of the products of both reactions showed two components, the minor having the retention time of the free steroid. Both products gave maximum ion pressures in the mass spectrometer at temperatures well below those required by the free steroids,

indicating the increased volatility. The spectra of both compounds showed the expected low intensity molecular ion (m/e 372) and base peak (m/e 143).

3.4.iii.[124] Trimethylsilylimidazole (3 drops) and trimethylchlorosilane (2 drops) were added to methandrostenolone (ca. 1mg) in a glass tube. The tube was stoppered and heated on a water bath at 80-90°C for several minutes. T.l.c. of the reaction mixture, from which there was no precipitation, indicated complete formation of the low polarity derivative.

3.4.iv.[125] Bistrimethylsilylacetamide (3.75µl), trimethylsilylimidazole (3.75µl) and trimethylchlorosilane (2.5µl) were added to the solid steroid (10-100µg) in a glass tube. The tube was stoppered and placed in an oven at 95-100°C for 30 minutes. n-Hexane (5 drops, ca. 0.1ml) was added to achieve a suitable concentration and the sample was injected into a g.l.c. instrument.

Both g.l.c. and t.l.c. indicated 100% silylation of methandrostenolone and its 17-epimer by this procedure.

3.5. The comparison of 17-epimethandrostenolone and the previously unidentified metabolite of methandrostenolone

The isolation and purification of samples of the metabolite (M) of methandrostenolone, each of which was estimated by carbon skeleton chromatography to contain about 160μg (3.5% of the dose of methandrostenolone) has been described in section 2. The analytical methods applicable to this size of sample were t.l.c., g.l.c. and mass spectrometry of the free and trimethylsilylated metabolite.

T.l.c. of several samples of M showed that each was a mixture of components, some of which gave different colour reactions with sulphuric acid from methandrostenolone and its 17-epimer. The majority of components in each sample were of similar polarity to each other and to the two epimeric standards in a solvent system (benzene-ethanol, 9:1) different from that with which the metabolic samples were isolated (chloroform-methanol, 9:1). Some of the components in this band gave the same colour reaction as the standards (orange-brown). No information was obtained from t.l.c. of a trimethylsilylated extract, probably because the concentration of the metabolite in the sample was too low.

G.l.c. of the metabolic samples on OV-1 at 220°C confirmed the presence of peaks at the retention time (16.2 minutes) of methandrostenolone or its 17-epimer. Chromatography, on OV-1 at 235°C, of similar samples after trimethylsilylation showed peaks at the retention time of 17-epimethandrostenolone trimethylsilyl ether (8.40 minutes) but none at the retention time of methandrostenolone trimethylsilyl

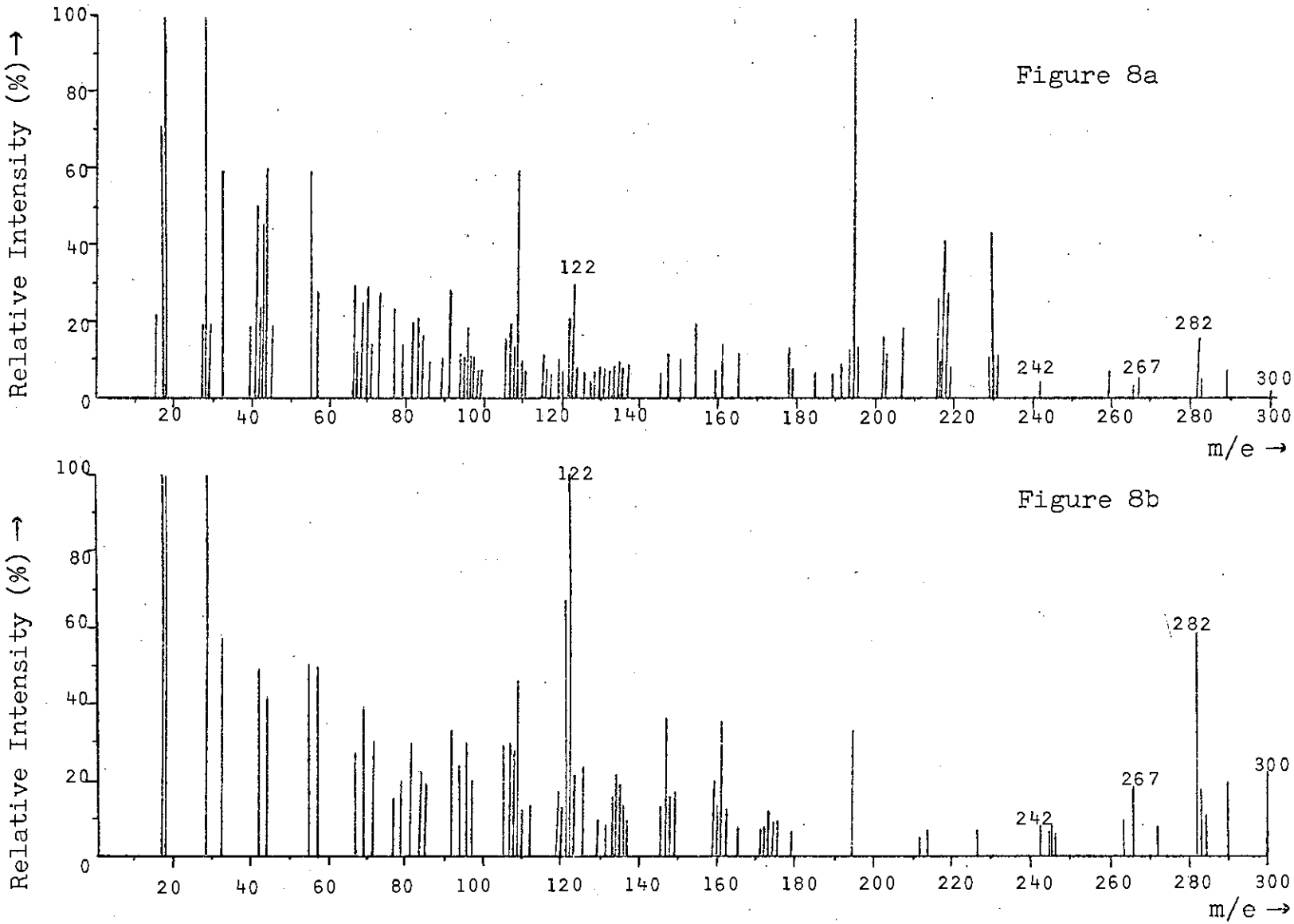


Figure 8. The mass spectra of two samples of the metabolite M, a) at 125°C and b) at 110° after preparative g.l.c.

ether (11.56 minutes).

Since mass spectra characteristic of methandrostenolone and its 17-epimer were obtained after preparative g.l.c. of the compounds this technique was used for additional purification of two samples of M prior to mass spectrometry. However, the mass spectra of these samples and of others which were not so purified contained very intense peaks at m/e 194 and m/e 109 at and below 90°C. At higher temperatures these ions, due to unknown impurities, declined and those at m/e 282 and m/e 122 increased. The spectra of samples of M [see figures 8a and 8b] contained all the characteristic and most intense ions of methandrostenolone and 17-epimethandrostenolone [see figures 5 and 7] as well as a variety of ions, below m/e 300, which did not belong to either of these compounds.

The ratios of the characteristic ions (m/e 300, m/e 282, m/e 267, m/e 242) [table 6] were similar to those of 17-epimethandrostenolone and differed from those of the 17β-ol [see tables 4 and 5]. Spectra of authentic methandrostenolone and its 17-epimer could not be obtained above 150°C because of low ion pressures. Similar behaviour of the ions corresponding to 17-epimethandrostenolone was observed in the spectra of metabolic extracts. The mass spectrum of the trimethylsilyl ether of M was similar to that of the derivative of 17-epimethandrostenolone with the molecular ion at m/e 372, the base peak at m/e 143 and an intense ion at m/e 282.

The examination of the free and trimethylsilylated metabolite M of methandrostenolone by gas chromatography and

Intensities (% base peak, m/e 122)	m/e300 M^+	m/e282 M-18	m/e267 M-18-15	m/e242 M-58	m/e282 / m/e300	m/e267 / m/e300	m/e242 / m/e267	Temp. (°C)
	7.7	53.8	15.4	15.4	7.0	2.0	1.0	90
Metabolite M	5.0	57.5	15.0	15.0	11.5	3.0	1.0	110
	4.5	54.5	15.9	15.9	12.1	3.5	1.0	125
	6.5	54.9	19.4	22.6	8.5	3.0	1.2	140
Mean ratios, Metabolite M					9.8	3.9	1.0	
	4.4	47.8	13.0	4.4	10.9	3.0	0.34	50
Metabolite M	6.3	52.1	14.6	6.3	8.3	2.3	0.43	70
after	3.9	44.5	14.5	5.6	11.4	3.7	0.39	80
preparative g.l.c.	21.6	58.9	15.7	5.9	2.7	0.73	0.38	90
	23.3	56.7	16.7	6.7	2.4	0.72	0.40	110
	47.6	57.2	19.1	9.5	1.2	0.40	0.50	140
Mean ratios, Metabolite M					6.2	1.8	0.41	

Table 6. The intensities (% base peak) of four characteristic ions in the spectra of samples of the metabolite M.

mass spectrometry thus identified it as 17-epimethandrostenolone by comparison with the authentic compound, synthesised by an unambiguous route. There was insufficient metabolic material for analysis by techniques which require larger samples but comparison with the melting point, infrared and elemental analysis data reported by Rongone and Segaloff[42] indicated that their less polar metabolite was also 17-epimethandrostenolone.

Schubert and Wehrberger[24] isolated twenty-one metabolites in their study of the metabolism of the 4-chloro analogue of methandrostenolone (Oral-Turinabol) in women. Four of these accounted for 92.2% of the material recovered as metabolites; the major metabolite (45.7%) was extracted from the "sulphate" fraction and identified as the unchanged drug. The 6β-hydroxy derivative (29.6%) was obtained from the free fraction and the 16β-hydroxy derivative (11.0%) from the glucuronide fraction. The fourth metabolite (5.9%) was isolated from the free fraction but it was not characterised. The remaining seventeen metabolites, none of which accounted for more than 2.5% of the total, accounted for the remaining isolated material (7.8%).

The unidentified metabolite (5.9%) from the free fraction was shown to possess the same A-ring as the drug itself. Its melting point (202-205°) was considerably higher than that of Oral-Turinabol (148-150°), a similar variation to that between methandrostenolone (164°) and its 17-epimer (221°). The chromatographic behaviour of the metabolite was not very different from that of the parent drug and its molecular weight, from mass spectrometry, was 334. The

molecular ion of Oral-Turinabol is m/e 335 but if the metabolite was the 17-epimer of the drug it might be expected to have a low intensity molecular ion which could have led to incorrect interpretation of the spectrum, of which no further details were reported. The chemical shifts of the methyl groups in the n.m.r. spectra of the metabolite and the drug were given[24] [see table 7] and show that although the C-19 and C-20 methyl signals were at similar positions in both compounds the C-18 methyl signal was shifted upfield by τ0.15 in the metabolite. An upfield shift of τ0.19 of the C-18 methyl resonance of 17-epimethandrostenolone compared to its 17-epimer was observed [table 7]. Consequently, it is possible that the unidentified metabolite of Oral-Turinabol may be the 17-epimer of the drug, although Schubert and Wehrberger[24] suggested that artefact formation might explain the n.m.r. spectrum. One of the seventeen minor metabolites also gave similar n.m.r. methyl shifts.

	Oral-Turinabol[24]	Metabolite of Oral-Turinabol[24]	Methandrostenolone	17-Epimethandrostenolone
C-19 methyl, τ	8.72	8.69	8.77	8.77
C-20 methyl, τ	8.85	8.79	8.82	8.81
C-18 methyl, τ	9.09	9.24	9.07	9.26

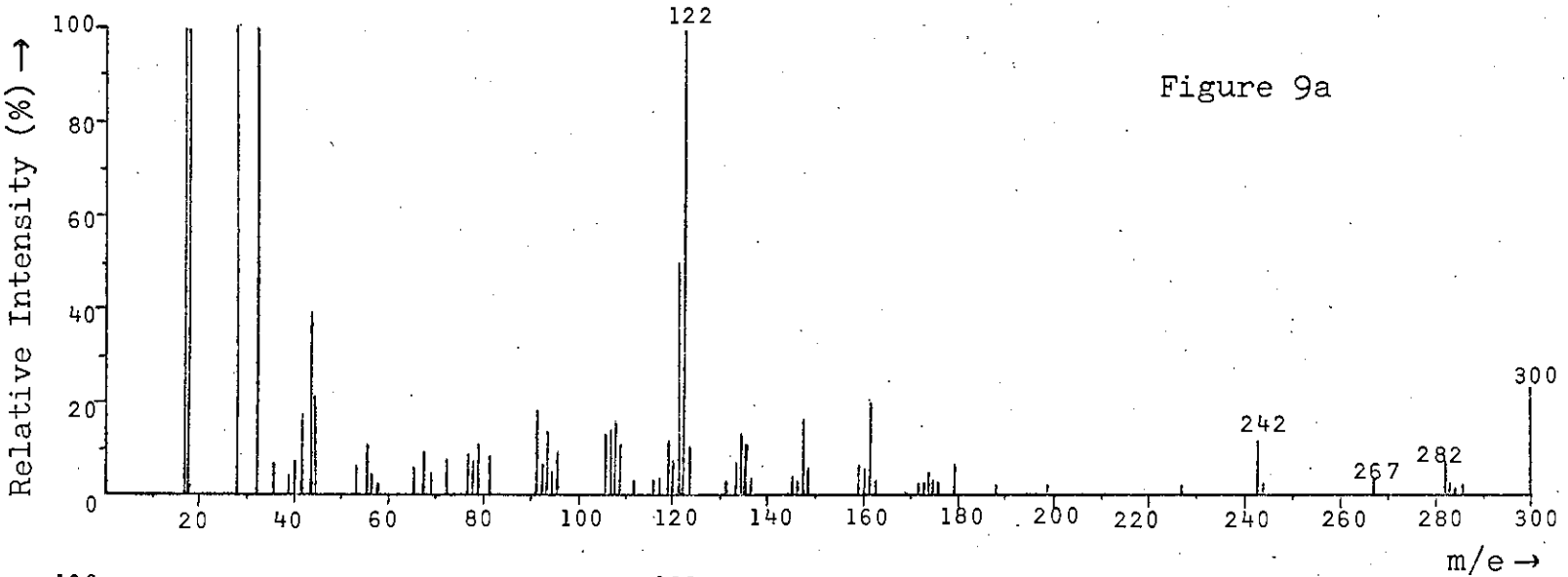
Table 7. The chemical shifts (τ values) of the C-18, C-19 and C-20 methyl groups of Oral-Turinabol, a metabolite of Oral-Turinabol, methandrostenolone and its C-17 epimer.

3.6. The examination of commercial methandrostenolone

Because only 3.5% of the dose of methandrostenolone could be isolated as 17-epimethandrostenolone it was necessary to establish that commercial methandrostenolone (Dianabol) does not contain a small percentage of the epimer. Methandrostenolone is prepared industrially by two routes, both of which require methyltestosterone as the starting material.[5c] The Δ^1- double bond may be introduced by chemical dehydrogenation or by specific microorganisms.

Methyltestosterone is synthesised by the reaction of a methyl Grignard reagent with the 17-keto group of dehydroepiandrosterone,[111b] a reaction which is almost totally stereospecific for the formation of the 17β-ol because of hindrance from the C-18 methyl group to attack from the β-side of the molecule.[111c,117] However 17-methylepitestosterone was isolated in very low yield during a large-scale synthesis of methyltestosterone.[87] In addition, it has been reported[142,143] that the 17β-hydroxy-17-methyl configuration is only formed in small yields when the molecule contains a 12α-hydroxyl group which hinders α-face attack. Similarly, mixtures of the 17a-hydroxy-17a-methyl epimers are formed in the Grignard reaction of a 17a-keto-D-homo steroid.[144]

The dehydrogenation of methyltestosterone has been accomplished by a variety of microorganisms[145] and there is no evidence to suggest that epimerisation might occur at this stage. However, if commercial methandrostenolone is free from the 17-epimer it must be concluded that mammalian metabolism is capable of accomplishing the 17-epimerisation reaction. In this case it is possible that a microbial



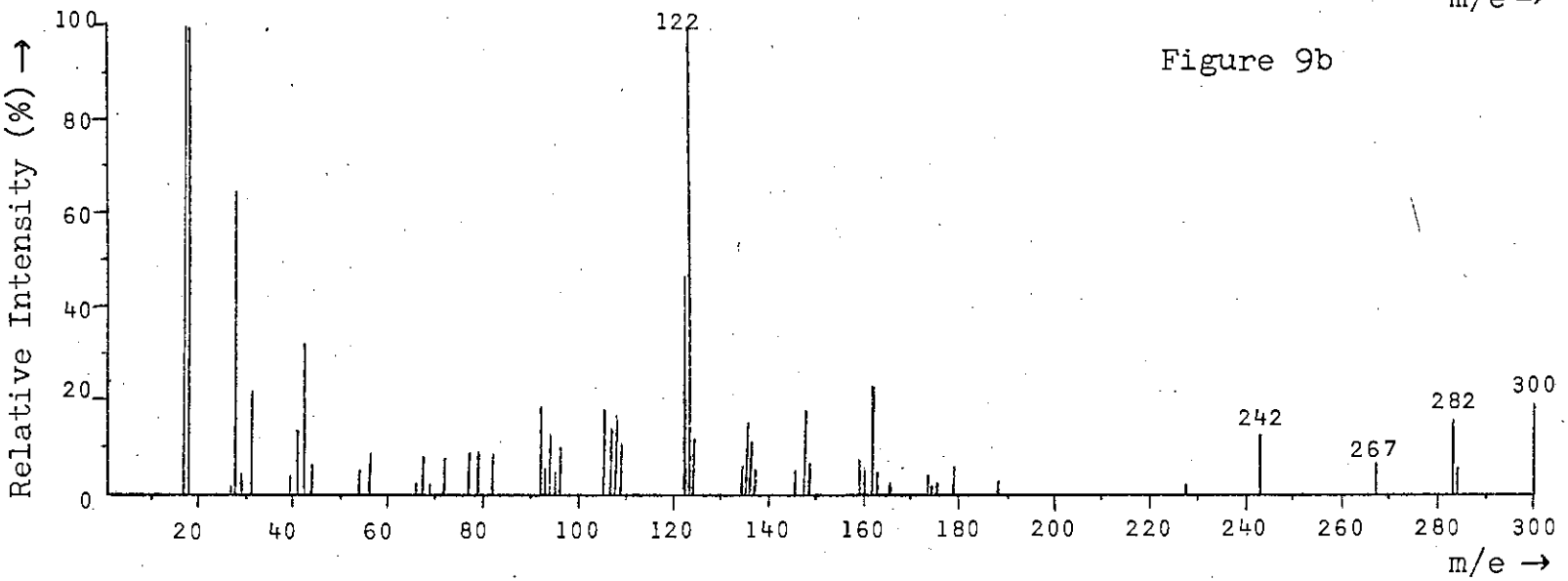


Figure 9. The mass spectra of a) tablet methandrostenolone and b) a mixture of methandrostenolone and its 17-epimer (95:5), both at 100°C.

enzyme system, able to carry out the transformation, could be found. Microbial introduction of the tertiary 17α-hydroxyl group is rare but has been reported[146] for progesterone, deoxycorticosterone and 11-dehydrocorticosterone with Tricothecium roseum and for progesterone with Trichoderma viride and with Dactylium dendroides. However these organisms are not used for the conversion of methyltestosterone to methandrostenolone.

Crystalline methandrostenolone extracted from tablets of Dianabol had the same melting point as authentic methandrostenolone and a mixed melting point was not depressed. The infrared and n.m.r. spectra were similar to those of methandrostenolone and g.l.c. of the trimethylsilyl ether gave no peak corresponding to the derivative of the 17-epimer. Mass spectra of methandrostenolone from tablets [figure 9a] were compared with those of the authentic material [figure 5a] and of mixtures of methandrostenolone and its 17-epimer (19:1)[figure 9b]. The spectra of tablet methandrostenolone were identical with those of the authentic compound whereas the mixtures showed small but significant differences, particularly in the ratio m/e 282/m/e 300 [see table 8]. Mixture 2, which gave ratios closer to those of pure methandrostenolone than did mixture 1, was known to contain less 17-epimethandrostenolone. The fact that the ratios m/e 282/m/e 300 and m/e 267/m/e 300 were greater for the mixtures than for the pure 17β-ol, and m/e 242/m/e 267 was less for the mixtures was predictable from a knowledge of the preferred fragmentations of both epimers.

Following this demonstration of the purity of commercial methandrostenolone material from this source was used in some of the experimental work to be described.

Intensities (% base peak, m/e 122)	m/e300 M^+	m/e282 M-18	m/e267 M-18-15	m/e242 M-58	m/e282 / m/e300	m/e267 / m/e300	m/e242 / m/e267	Temp. (°C)
Tablet Methandrostenolone	24.2	8.1	4.0	12.9	0.33	0.17	3.2	100
	19.5	6.3	3.7	10.9	0.32	0.18	3.0	110
	20.0	7.5	3.8	11.6	0.38	0.19	3.1	110
	28.6	8.6	5.7	14.3	0.30	0.20	2.5	120
	19.1	7.1	3.6	11.9	0.37	0.19	3.3	120
	11.0	3.0	2.0	6.0	0.27	0.18	3.0	130
	22.2	8.3	4.2	13.9	0.37	0.19	3.3	130
	40.3	16.7	8.3	29.9	0.41	0.21	3.6	140
	50.0	20.9	10.5	24.4	0.42	0.21	2.3	145
	16.0	5.0	3.5	11.0	0.31	0.22	3.1	150
	7.5	3.0	2.0	6.0	0.40	0.27	3.0	180
	11.4	5.7	5.7	11.4	0.50	0.50	2.0	200
Mean ratios, tablet methandrostenolone					0.37	0.23	3.0	
Methandrostenolone + 17-Epimethandrostenolone (ca. 19:1) Mixture 1	7.0	8.5	2.5	3.0	1.2	0.36	1.2	90
	11.0	16.5	6.0	7.0	1.5	0.55	1.2	100
	11.7	18.1	6.4	8.5	1.5	0.55	1.3	125
	18.6	28.9	12.4	14.4	1.6	0.65	1.2	180
Mean ratios, mixture 1					1.5	0.53	1.2	
Methandrostenolone + 17-Epimethandrostenolone (ca. 19:1) Mixture 2	30.0	13.3	5.0	15.0	0.44	0.17	3.0	85
	19.7	16.4	6.6	13.1	0.85	0.34	2.0	100
	15.0	12.0	4.0	9.0	0.80	0.27	2.3	110
	24.5	17.0	6.0	15.5	0.69	0.25	2.6	120
	38.0	18.0	8.5	26.5	0.47	0.22	3.1	130
Mean ratios, mixture 2					0.65	0.25	3.3	

Table 8. The intensities (% base peak) of four characteristic ions in the mass spectra of tablet methandrostenolone and of mixtures of methandrostenolone and its 17-epimer.

3.7. Precursor-product studies on the metabolism of methandrostenolone

The conjugation of steroids with glucuronic or sulphuric acids and excretion of the conjugates in the urine is a major pathway in the metabolism of natural and synthetic compounds.[21,147] The metabolism of methandrostenolone is unusual both because the two major metabolites are excreted as free steroids[32] and because there is no biochemical precedent for the epimerisation of a tertiary hydroxyl group. The stability of the $\Delta^{1,4}$-dien-3-one system towards biochemical transformations has been reported as a result of investigations of the metabolism of a variety of drugs with this functional group.[24,26,50] In view of this stability the obvious mode of metabolism of methandrostenolone is hydroxylation, which occurs to give the 6β-hydroxy derivative.

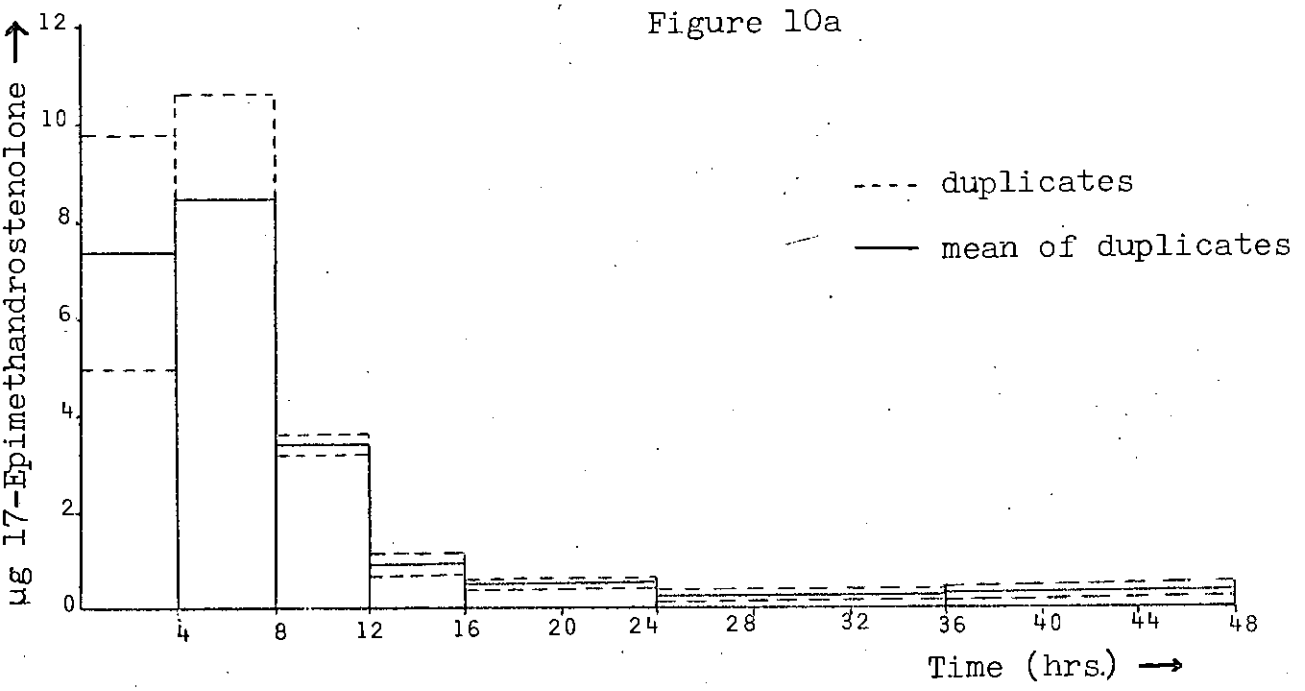
Since the few 17α-hydroxy epimers of androgenic and anabolic steroids which have been tested were found to possess very little biological activity identification of the 17-epimer of methandrostenolone as the major metabolite of the drug in normal men suggests that mammalian metabolism is capable of detoxifying an otherwise exceptionally stable drug by formation of the virtually inactive 17α-ol. The metabolism of very few synthetic steroid drugs with the 17β-hydroxy-17-methyl function has been studied and it is probable that the "epimerisation pathway" has not been observed before because most of these compounds, unlike methandrostenolone, contain other substituents which are more readily metabolised by conventional pathways.

It is probable that the epimerisation of methandrosten-

olone may be accomplished by the action of one or more enzymes. Confirmation of this would require isolation of the purified "epimerase" and verification of its specificity and activity by in vitro experiments which was impracticable during this study. Consequently the problem of the mechanism of epimerisation was approached by the assumption of the existence of an "activated intermediate". This intermediate could then be sought in the urine following administration of methandrostenolone and the epimerisation attempted by chemical synthesis and reaction.

Sulphate conjugates of steroids are abundant and yet no such conjugates of the metabolites of methandrostenolone could be isolated. Consequently the possibility that methandrostenolone sulphate might be an intermediate in the 17-epimerisation reaction was considered. The sulphate group is a good leaving group and attack by a hydroxyl group from the α-face might thus lead to 17-epimethandrostenolone. The object of the precursor-product studies was to examine whether methandrostenolone sulphate was produced and then converted to the epimer, in which case the sulphate would only be isolated soon after administration of the drug.

Urine was collected at intervals of four hours up to sixteen hours after the administration of 10mg of methandrostenolone and then at one eight hour and two twelve hour intervals up to forty-eight hours. The urine collections were halved to provide duplicates and each was extracted with its own volume of chloroform to give the free fraction containing the product, 17-epimethandrostenolone, as described previously (section 2). Pyridinium sulphate was then added to the



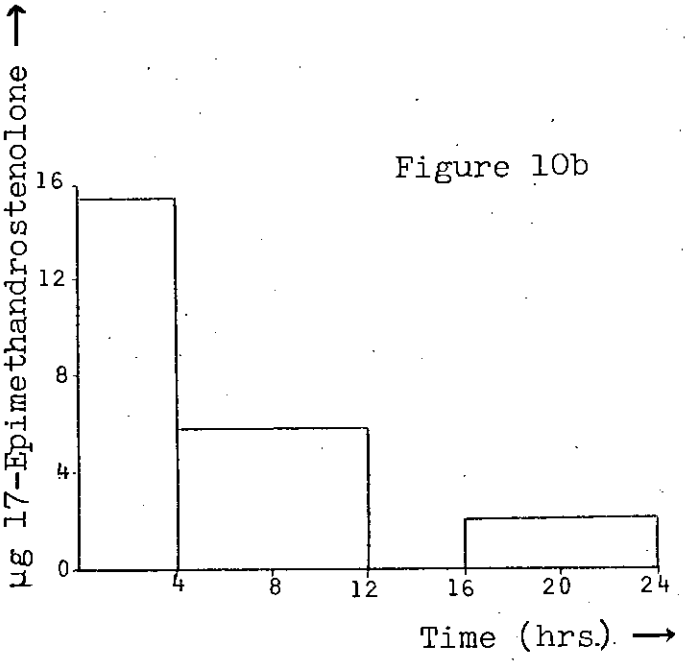


Figure 10. Histograms showing the variation of the weight of 17-epimethandrostenolone extracted from urine with time after the administration of methandrostenolone.

aqueous phase to form the pyridinium salts[147c] of any steroid sulphates which might be present. These were extracted with chloroform and hydrolysed with hydrochloric acid to give the sulphate fraction which might contain a precursor, methandrostenolone sulphate. A second study was carried out in which urine was collected for twenty-four hours but duplicates were not performed.

The free and sulphate fractions were examined by carbon skeleton chromatography for steroids with the same polarity as methandrostenolone and bearing the 17-methyl substituent. The material in these bands was then analysed by quantitative g.l.c. with pregnanediol diacetate as an internal standard.

The histograms [figures 10a and 10b] show the approximate weights of 17-epimethandrostenolone found in the free fractions in each successive four hours after administration of the drug. The correspondence between the two duplicates [broken lines, figure 10a] in the first two four hour periods was poor, although in the following samples it was good. However, both studies showed that maximum excretion of 17-epimethandrostenolone occurred within the first eight hours. In a study of the metabolism of 17α-^{14}C-methandrostenolone in the dog Sandor and Lanthier[45] found that the bulk of the urinary metabolites were excreted unconjugated in the second twenty-four hours following intravenous injection. The different modes of application of the drug probably largely account for this difference in excretion rates.

In the first study a sulphate fraction corresponding to each free fraction was analysed. The majority of these

fractions did not contain a component with the same retention time as methandrostenolone and although several of the earliest fractions gave a small peak at this retention time the quantity of material was insufficient to establish the identity of the peak by silylation. After individual analysis the fourteen sulphate fractions were combined and re-chromatographed to give a peak (ca. 11µg) which could have been one of the epimers of methandrostenolone. The total sulphate fraction from the second study was analysed and gave a peak corresponding to only 0.33µg of material at the retention time of methandrostenolone.

These results failed to demonstrate the existence of methandrostenolone sulphate and hence a precursor-product relationship between this conjugate and 17-epimethandrostenolone could not be found. To demonstrate such a relationship would have required the extraction of material with the retention time of methandrostenolone from the sulphate fraction, such that the histogram of extracted steroid against time reached a maximum prior to the corresponding histogram of 17-epimethandrostenolone extracted from the free fraction. Because 17-epimethandrostenolone was found to be excreted so rapidly urine collections would need to be made at very short intervals during the first eight hours after administration in order to observe a maximum in the sulphate fraction prior to that in the free fraction if a precursor-product relationship did exist.

An experiment was carried out to determine whether small quantities of free steroids might be carried over into the sulphate fraction by partition of chloroform in water during

the initial chloroform extraction of free steroids. 17-Epimethandrostenolone (130µg) was partitioned between equal volumes of chloroform and water, the chloroform layer discarded and the aqueous layer subjected to the "sulphate extraction procedure".[147c] The final residue was trimethylsilylated and examined by t.l.c. which showed at least seven components, one of which was of low polarity and gave a similar colour reaction to methandrostenolone and its 17-epimer. G.l.c. indicated that the mixture contained seven components, one with a retention time similar to that of 17-epimethandrostenolone trimethylsilyl ether. Consequently, the very small peaks at the retention time of methandrostenolone in the sulphate fractions may have been 17-epimethandrostenolone carried over from the free fraction.

3.8. Experimental Section

3.8.i. Precursor-product studies

Methandrostenolone (10mg) was taken orally in the morning and urine collected at 4 hour intervals for the first 16 hours, then after an interval of 8 hours and finally at 12 hour intervals up to 48 hours after administration of the drug. Each urine collection was halved to provide duplicates and both portions were extracted with chloroform to give the free steroids and then treated by a procedure designed to extract and hydrolyse any steroids which might be present as the sulphate conjugates.[147c] Those 17-methylandrostane components, with similar polarity to methandrostenolone, in both fractions were identified by carbon skeleton chromatography and isolated as described previously (section 2). The residues from the 'free fraction' and the 'sulphate fraction' were then examined by g.l.c.

Chromatography was carried out on a Pye 104 gas chromatograph with glass columns packed with 2½% OV-1 stationary phase and operated at 235°C with a nitrogen flow rate of 40ml per minute. Free methandrostenolone gave an asymmetric peak because of tailing caused by the hydroxylic nature of the compound and its low volatility. The acetates of small samples of primary and secondary steroid alcohols may be conveniently prepared for g.l.c. by exposure of the free steroids to a dry atmosphere of pyridine and acetic anhydride but much harsher conditions are necessary for the synthesis of the acetates of tertiary alcohols (see section 3.13.iv). When this piece of work was carried out trimethylsilylation was unsatisfactory because the method

employed (see section 3.4.ii) did not result in 100% formation of the derivative and the background, when the silylated metabolic samples were chromatographed, was very high. Consequently, samples were analysed without prior derivative formation; after analysis of the free metabolite the trimethylsilyl derivative of one fraction was prepared and chromatographed to identify the epimer present in the sample. A peak with the retention time of 17-epimethandrostenolone trimethylsilyl ether was obtained. Although free methandrostenolone did not give a symmetrical peak good agreement was obtained when methandrostenolone solutions of known concentration were compared with similar solutions of standard compounds with an electronic integrator and by the weights of the cut-out peaks.

Pregnanediol diacetate was used as an internal standard for the quantitative analysis of the metabolic samples. It had a longer retention time than the epimers of methandrostenolone and chromatographed well at 235°. Compounds with shorter retention times were examined but were unsuitable because of the presence of contaminants with short retention times in the metabolic samples.

The inside of each tube containing a metabolic residue was washed with a small volume of dry ether and carefully evaporated to dryness with nitrogen, to concentrate the sample in the bottom of the tube. Pregnanediol diacetate (ca. 2mg) was weighed accurately on a Cahn electrobalance, transferred to a graduated flask and made up to 100ml with Analar acetone. An aliquot of this solution (0.5ml or 0.25ml, containing ca. 10µg or 5µg, respectively, of the standard) was added from a

Sample	Time after administration (hours)	x	y	$\frac{x}{y}$	Y(μg)	$X=Y\frac{x}{y}$ (μg)	X per 4 hours	Mean X per 4 hours
Free fraction, 17-epimethandrostenolone								
1	0-4	36018	70347	0.499	9.77	4.88	4.88	7.29
2	0-4	46735	47065	0.993	9.77	9.70	9.70	
3	4-8	6058	6001	1.011	10.415	10.53	10.53	8.39
4	4-8	437	728	0.600	10.415	6.25	6.25	
5	8-12	2479	7662	0.323	10.415	3.36	3.36	3.17
6	8-12	1755	6157	0.285	10.415	2.97	2.97	
7	12-16	1798	16086	0.112	10.415	1.17	1.17	0.93
8	12-16	1038	15856	0.065	10.415	0.68	0.68	
9	16-24	1870	27033	0.069	10.415	0.72	0.36	0.40
10	16-24	1073	6496	0.165	5.2075	0.86	0.43	
11	24-36	1919	14322	0.134	5.2075	0.70	0.23	0.14
12	24-36	48	2351	0.024	5.2075	0.12	0.04	
13	36-48	2606	17965	0.145	5.2075	0.76	0.25	0.17
14	36-48	483	9167	0.053	5.2075	0.28	0.09	
Sulphate fraction, material with retention time of methandrostenolone								
1	0-4	200	5807	0.035	10.415	0.37	0.37	0.19
2	0-4	11	5587	0.002	5.2075	0.01	0.01	
3	4-8	122	6805	0.018	5.2075	0.09	0.09	0.09
4	4-8	-	1672	-	5.2075	-	-	
5	8-12	-	11018	-	5.2075	-	-	0.02
6	8-12	27	9761	0.003	5.2075	0.02	0.02	
7	12-16	630	11475	0.055	5.2075	0.29	0.29	0.29
8	12-16	-	8880	-	5.2075	-	-	
9	16-24	-	9924	-	5.2075	-	-	
10	16-24	-	4193	-	5.2075	-	-	
11	24-36	-	9186	-	5.2075	-	-	
12	24-36	-	8107	-	5.2075	-	-	
13	36-48	-	7159	-	5.2075	-	-	
14	36-48	-	9975	-	5.2075	-	-	

Table 9. Results of the analysis of the "free" and the "sulphate" fractions from urine following the administration of methandrostenolone

burette to the tube containing the residue to be analysed and the solvent removed by careful evaporation with nitrogen at room temperature.

The residue, containing Xμg of the compound to be analysed and Yμg of the standard was then dissolved in a small volume (ca. 10μl) of Analar acetone and injected into the gas chromatograph. The areas of the peaks, x and y, corresponding to the unknown and to the standard, respectively, were measured with a Kent Chromalog 2 Integrator and X was determined from the proportional relationship:- $\frac{x}{y} = \frac{X}{Y}$

Sample	Time after administration (hours)	x	y	$\frac{x}{y}$	Y(μg)	$X=Y\frac{x}{y}$ μg	X per 4 hours
		Free fraction, 17-epimethandrostenolone					
1	0-4	3466	2128	1.63	9.2	15.0	15.0
2	4-8	2699	4254	0.635	9.2	5.8	5.8
3	8-12	3546	5594	0.635	9.2	5.8	5.8
4	12-16	-	3926	-	9.2	-	-
5	16-24	2267	4810	0.472	9.2	4.3	2.2
		Sulphate fraction, material with retention time of methandrostenolone					
1-5 (inc.)	0-24	347	9703	0.036	9.2	0.33	

Table 10. Results of the analysis of the "free" and the combined "sulphate" fractions from urine following the administration of methandrostenolone

Tables 9 and 10 show the results of two studies which are represented in histogram form in figures 10a and 10b. Fraction 4, table 10 was examined twice and in both cases no material with the retention time of methandrostenolone was found.

3.8.ii. Treatment of 17-epimethandrostenolone by the "sulphate extraction procedure"[147c]

17-Epimethandrostenolone (131µg) was ground to a fine powder and washed into a separating funnel with water (300ml) and chloroform (300ml). The mixture was shaken well and the chloroform layer discarded. The aqueous layer was then made 0.3M in pyridinium sulphate [pyridine (16ml) and concentrated sulphuric acid (5.4ml)] and extracted with chloroform (320ml). The aqueous layer was discarded and the organic layer washed with distilled water (2 x 32ml), dried over anhydrous magnesium sulphate and evaporated to dryness. The residue was dissolved in water (10.9ml) and concentrated hydrochloric acid (0.1ml) was added to the solution, which was left to stand for 5 minutes before extraction with chloroform (2 x 11ml). The combined organic extracts were washed with 1N sodium hydroxide solution (11ml) and with water (2 x 11ml), dried over anhydrous magnesium sulphate and evaporated to dryness to give a brown residue. The trimethylsilyl derivative of the residue was prepared according to the method described in section 3.4.iv. T.l.c. of this derivative showed seven spots, the least polar of which gave the colour reaction of methandrostenolone and its 17-epimer. Seven components with retention times between 6.4 and 23.8 minutes

on 2½% OV-1 at 235°C were detected by g.l.c. One of the components had a retention time (11.4 minutes) similar to that of the trimethylsilyl ether of 17-epimethandrosten-olone.

3.9. The investigation of compounds which might be intermediates in the epimerisation of methandrostenolone

3.9.i. It has been shown (section 3.7) that material resembling methandrostenolone sulphate could not be isolated from the urine, following administration of methandrostenolone, in more than negligible quantities, which may have been due to carry-over of free steroid rather than to extraction and hydrolysis of a sulphate. If methandrostenolone sulphate existed but was unstable to the extraction procedure methandrostenolone should have been detected in the free fraction because chemical hydrolysis of a sulphate, with few exceptions, leads to fission of the O-S bond and retention of configuration rather than to fission of the C-O bond with inversion.[148]

There is a possibility, however, that a transitory sulphate intermediate might exist. The enzymes responsible for the sulphation of steroids, the steroid sulphotransferases, have been found in significant amounts only in the liver, adrenals and jejunal mucosa in the adult human,[149] whilst those which catalyse the hydrolysis of steroid sulphates, the steroid sulphatases, are known to occur in the liver and adrenals as well as the testes and ovaries.[150] Consequently, if enzymes of these types are involved in the epimerisation of methandrostenolone it is possible that a sulphate might exist transitorily in organs in which both types of enzymes are found.

The epimerisation of a tertiary hydroxyl group is such an unusual reaction that, although very little can be said about the mechanism of the biological reaction without

enzyme studies, it was of interest to explore the possibility of effecting such a transformation by chemical means. Originally it was considered[151] that steroid sulphates were final products of metabolism but several workers have now shown that these conjugates can participate in a variety of biochemical reactions.[151,152] It has been demonstrated that the conversion of cholesterol and pregnenolone to biologically active steroids may take place at the level of the sulphate esters.[153-156] Consequently the synthesis of methandrostenolone sulphate was attempted.

3.9.ii. Methods of synthesis of steroid sulphates

Many methods have been developed for the synthesis of steroid sulphates following the appreciation of their importance as products of metabolism. Three reactions, in each of which the active sulphating agent is the pyridine-sulphur trioxide complex, have formed the basis for the majority of these syntheses.[157,158] The complex may be generated *in situ* by the reaction of chlorosulphonic acid with pyridine, and the steroid to be sulphated simply added to the reaction. This method has been widely used[157] but suffers from the disadvantage that it is sometimes difficult to remove chloride ions completely from the final products.[159] An alternative method involves the preparation of pyridine sulphate and the use of an internal dehydrating agent, usually acetic anhydride, to generate the pyridine-sulphur trioxide complex.[157] This method is effective provided that the concentration of acetic anhydride relative to pyridine sulphate is not too great, in which case the steroid acetate is the primary product.[160] A

third method for the generation of pyridine-sulphur trioxide involves the direct reaction of solid sulphur trioxide with pyridine in chloroform. This reaction is rather violent and has not been much used because of the difficulty of handling sulphur trioxide.[157] A method which utilises sulphamic acid as the sulphating reagent has been described by Joseph *et al.*[161]

Methods of steroid sulphation which utilise the isolated adduct of sulphur trioxide with a nitrogenous base are convenient because of the absence of chloride ions. The preparation of pyridine-sulphur trioxide was described by Baumgarten[162] in 1926 and its properties and reactions have been reviewed by Gilbert.[163] A commercial preparation of pyridine-sulphur trioxide, m.p. 141-143°, was initially employed in the work to be described and Roy and Trudinger[159] described a convenient method of preparation of the complex, m.p. 175°. In view of the discrepancy between these reported melting points material was synthesised according to the latter method and found to have a melting point of 140°. Melting points ranging between 97° and 175°, depending on the method of preparation, have been reported[163] for pyridine-sulphur trioxide. Gilbert[163] has explained these discrepancies in terms of the salt-like nature of the complex, its low volatility and low solubility in all common solvents making purification exceeding difficult.

Roy and Trudinger[159] stated that the method which they described for the preparation of pyridine-sulphur trioxide was also suitable for the synthesis of the adduct of sulphur trioxide with other bases, such as trimethylamine.

Consequently the synthesis of triethylamine-sulphur trioxide was attempted by this method. Unlike the pyridine adduct, the product of the addition of chlorosulphonic acid to triethylamine was soluble in chloroform and so anhydrous ether was added to achieve precipitation. The white crystalline material was recovered by filtration and purified according to the method, described by Dusza et al,[164] for the purification of commercial triethylamine-sulphur trioxide. However, the purified product had a melting point above 250° whereas that of triethylamine-sulphur trioxide is 92°. Hence triethylammonium salts of steroid sulphates were prepared by the addition of triethylamine to reaction mixtures containing pyridine-sulphur trioxide. Because of the greater basicity of triethylamine it readily replaces pyridine as the cation.

Several other methods have been described for the preparation of steroid sulphate esters. That of Mumma et al.[165,166] which utilises dicyclohexylcarbodiimide for the generation of the active sulphating species from sulphuric acid was used for an attempted sulphation of methandrostenolone, described later.

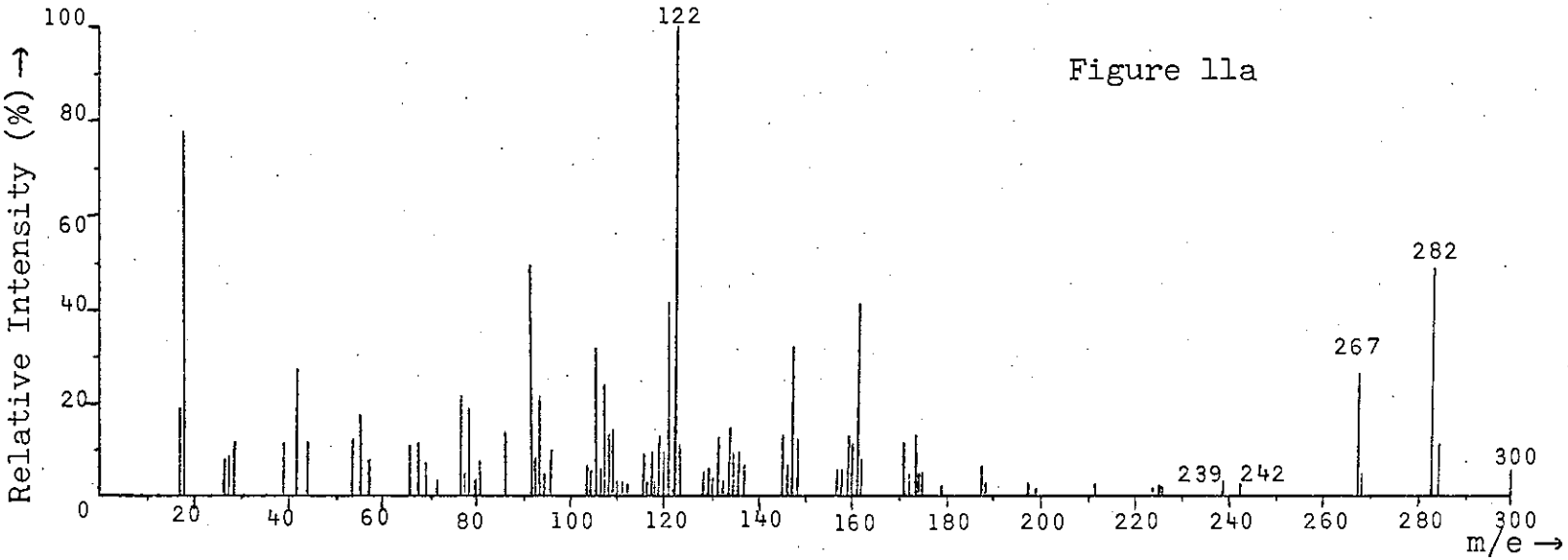
The literature contains many references to methods suitable for the preparation of sulphates of primary and secondary steroid hydroxyl groups, but there are few references to the sulphation of tertiary steroid alcohols.[167] Dusza et al.[164] were able to synthesise the sulphates of the tertiary 17α-hydroxyl group of 17α-hydroxyprogesterone and related compounds by a method employing triethylamine-sulphur trioxide. Joseph et al.[161] have prepared the sulphates of

the 17β-hydroxyl group of 17α-ethynyl-3-methoxyoestra-1,3,5(10)-trien-17-ol and 17α-ethynyloestra-1,3,5(10)-triene-3,17-diol with sulphamic acid. However reports of tertiary 17β-sulphates in which the 17α-substituent is an alkyl group are more scarce.[167] D'Alo[168] prepared the disodium salt of 17α-methylandrost-5-ene-3β,17-diol disulphate, which compound was found to be unstable at ambient temperature and only stable below 8°. It has long been known that steroids can be biochemically sulphated at the 3,17 and 21 positions[169-171] but Wengle and Boström[172] have shown that the introduction of a methyl or ethyl group at the 17α-position inhibits the sulphation of the 17β-hydroxyl group.

3.9.iii. The synthesis of sulphates of methandrostenolone

The properties of the triethylammonium salts of steroid sulphates make them desirable derivatives compared to some of the more common salts.[164] Because of the apparent failure of attempts to synthesise the triethylamine-sulphur trioxide complex, salts of triethylamine were prepared by exchange with pyridinium salts. Several variations of the basic method of preparation of the steroid sulphate from pyridine-sulphur trioxide and triethylamine were employed in attempts to synthesise the derivative of methandrostenolone.

Sulphates are very polar compounds and would be expected to have exceedingly low R_f values in the solvent systems normally employed for t.l.c. of the less polar free steroids. Infrared spectra of steroid sulphates in potassium bromide discs have often been recorded but a variety of solvents may be used for solution spectra provided that a suitable cation is used to confer solubility.[147a] Intense absorption bands,



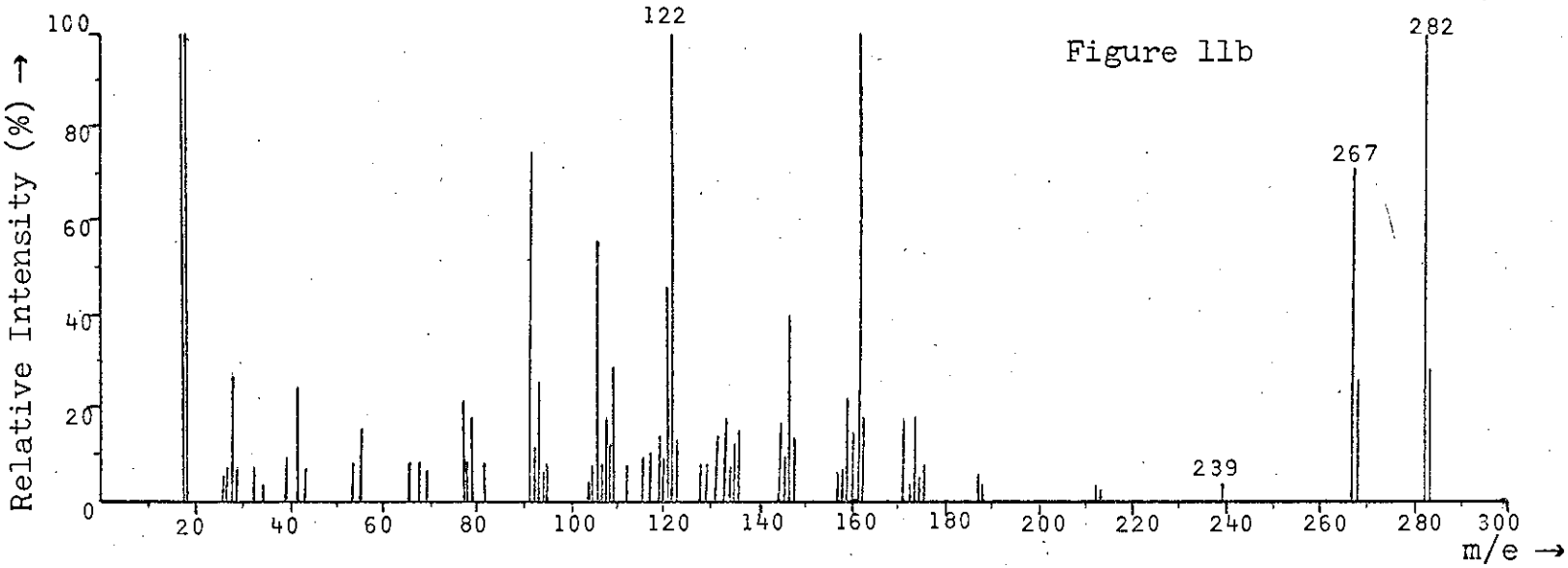


Figure 11. The mass spectra of a) the products of the sulphation of methandrostenolone and b) 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one.

characteristic of the sulphate moiety, occur at ν_{max}1240 and 1225cm^{-1}, sometimes not fully resolved because of their intensity, and at ν_{max}1070-1060cm^{-1}.[147a,173] A series of intense bands, characteristic of the S-O-C bonds and thus dependent on the nature of the steroid appear in the region ν_{max}1050-650cm^{-1}.[173]

Methandrostenolone was reacted with pyridine-sulphur trioxide and triethylamine in pyridine for two hours at room temperature. The addition of ether gave a slight precipitate which could not be recovered by filtration; consequently the solvents were removed by evaporation and the resulting crystals washed with ether. Emiliozzi[174] described the isolation of androsterone triethylammonium sulphate by distillation of the solvents in vacuo below 60°. The product of this reaction of methandrostenolone was initially examined by t.l.c. and infrared spectroscopy, both of which indicated that no reaction had occurred.

The n.m.r. spectrum of the same product showed insignificant shifts of the methyl peaks from the positions of those of the free steroid. However, the spectrum included a triplet at τ8.65 (J = 7Hz) and a quartet at τ6.84 (J = 7Hz) which integrated for 20% of the sample. These resonances were neither free triethylamine [τ9.03 (t, 7.2 Hz), 7.57 (q, 7.2 Hz)] nor diethyl ether [τ8.81 (t, 7.2 Hz), 6.52 (q, 7.2 Hz)] but were similar to the positions reported[164] for the ethyl protons in steroid triethylammonium sulphates [τ8.67 ± 0.08(t), 6.92 ± 0.08 (q)]. The mass spectrum of the product [figure 11a] was different from that of methandrostenolone [figure 5a], particularly in the appearance of a meta-

stable ion at m/e 252.7, attributable to the transition, m/e 282 to m/e 267, the near disappearance of m/e 242 and the appearance of a low intensity ion at m/e 239.

The sulphate of methandrostenolone was also prepared by the method of Emiliozzi,[174] as described by Roy and Trudinger,[159] in which the reactants were heated to give a solution prior to the addition of triethylamine. Once again, examination of the product by t.l.c. and infrared spectroscopy suggested that it was unreacted methandrostenolone but the mass spectrum was similar to that of the product of the previous reaction [figure 11a]. The figures obtained from elemental analysis were not in exact agreement with those calculated for methandrostenolone triethylammonium sulphate but were of the correct order. Hydrolysis of this product in an aqueous medium containing a trace of base gave a compound with the mass spectrum, n.m.r. spectrum and melting point of methandrostenolone.

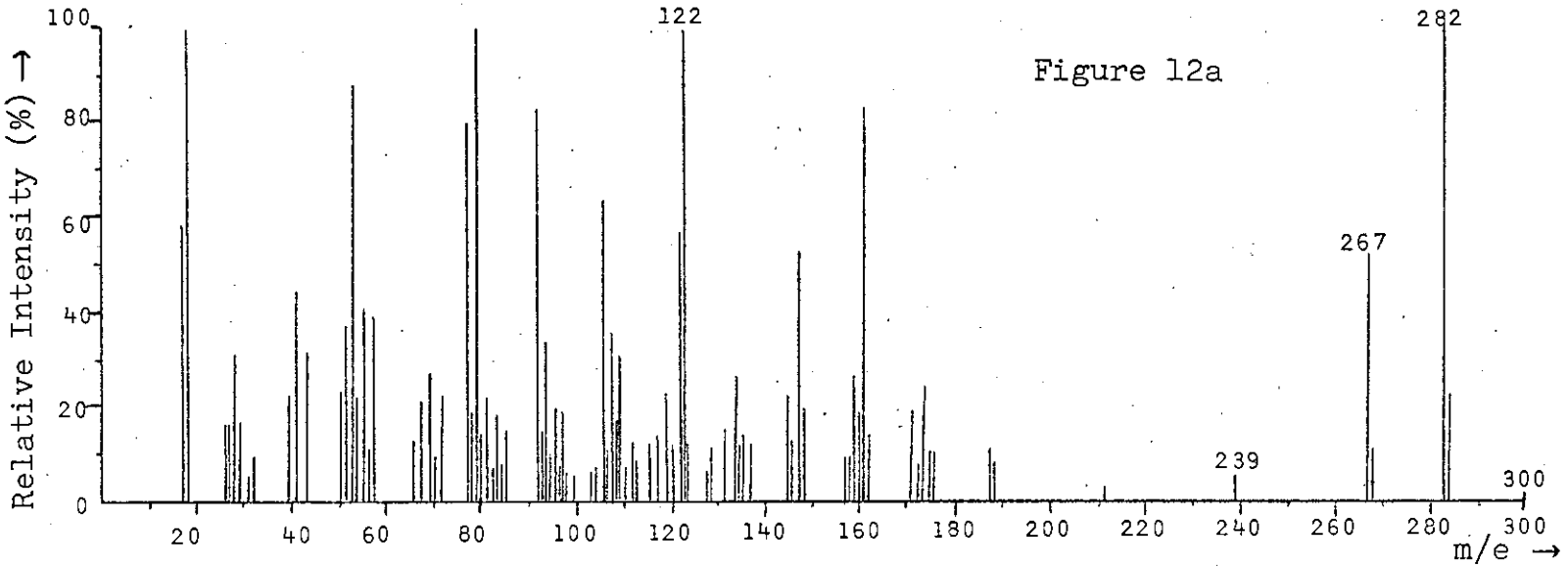
Finally a procedure recommended for the formation of the triethylammonium sulphates of steroids with hindered hydroxyl groups,[164] in which the reactants were heated at 70-90^{o} for three hours was examined. The ethyl protons in the n.m.r. spectrum of a sample of methandrostenolone triethylammonium sulphate prepared by this method were twice as intense as was required for 100% formation of the derivative. This was explained by the fact that chemical exchange occurred between the ammonium proton of the salt and any free triethylamine which had not been removed by evaporation. Consequently, all the ethyl protons, whether in the cation or the free base

appeared at the resonance positions of the cation, and the ammonium proton appeared as a very broad band in the region τ3.6 - 2.8. Thus, unless all free triethylamine was removed n.m.r. spectroscopy could not be used to calculate the percentage formation of the triethylammonium sulphate. The pyridinium salt of methandrostenolone sulphate was prepared and gave a mass spectrum similar to the products of the previous sulphation reactions.

3.9.iv. Interpretation of the results of the sulphations of methandrostenolone

Rationalisation of the results of the experiments described in the above section was difficult because information from the different methods of examination of the products appeared to conflict. In all cases t.l.c. and infrared spectroscopy indicated that no reaction had occurred, as did the lack of any shift of the methyl signals, particularly the C-18 methyl resonance, in the n.m.r. spectra. However the appearance of the quartet and triplet at the reported chemical shifts[164] of the triethylammonium moiety, and the elemental analyses were indicative of successful formation of methandrostenolone sulphate. Mass spectrometry provided the most conclusive evidence for formation of a derivative, since each product gave a similar spectrum which was not that of free methandrostenolone. The recovery of methandrostenolone after hydrolysis of the derivatives eliminated the possibility that decomposition might have occurred prior to mass spectrometry.

The literature contains little concerning the mass



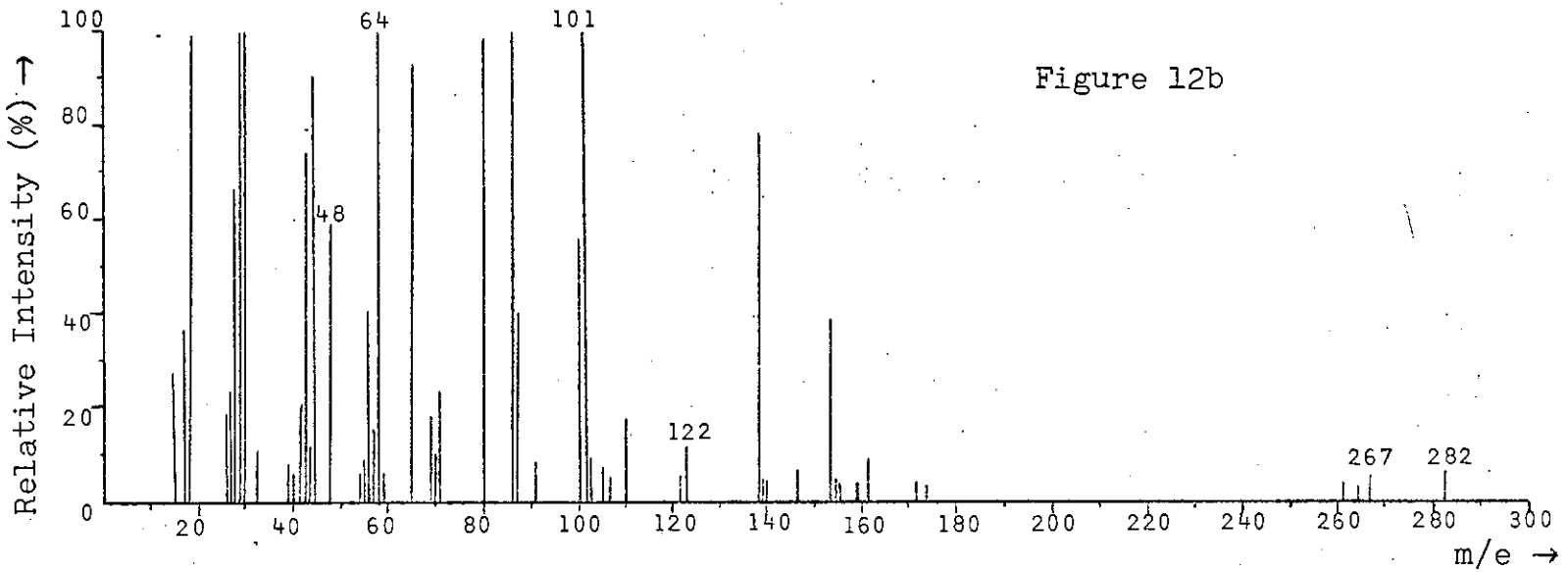
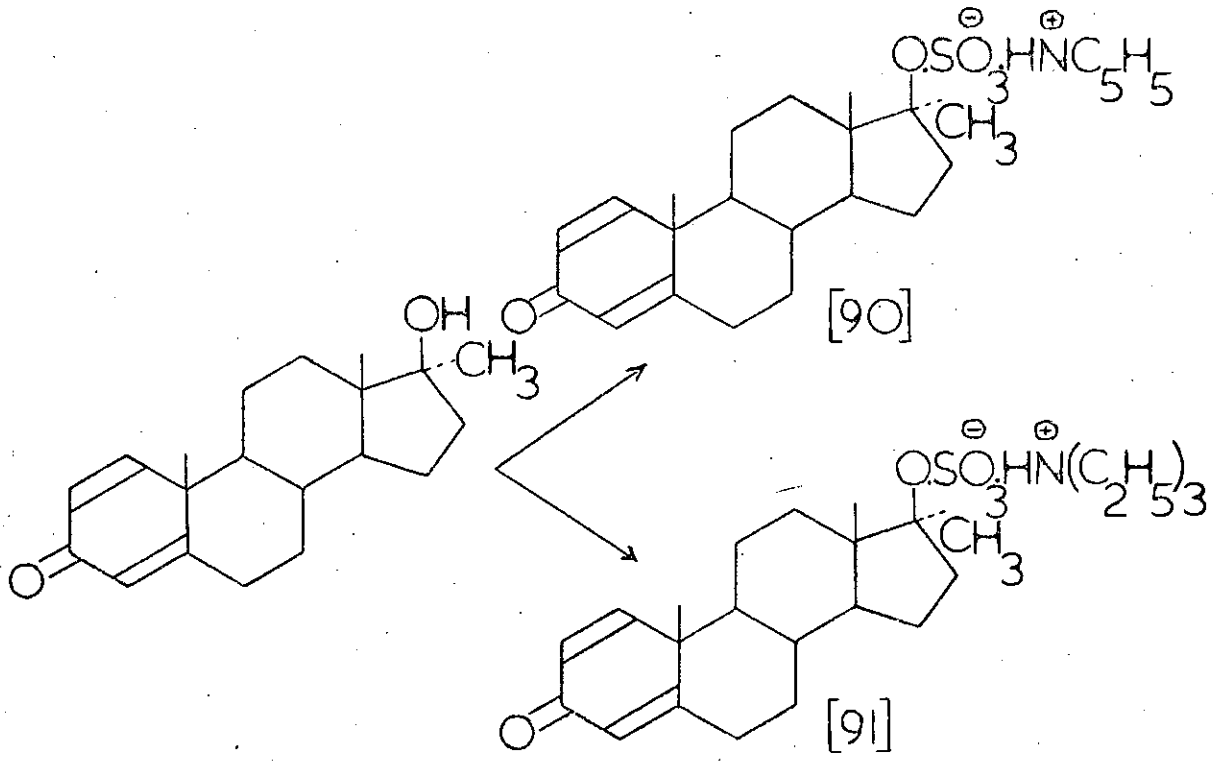
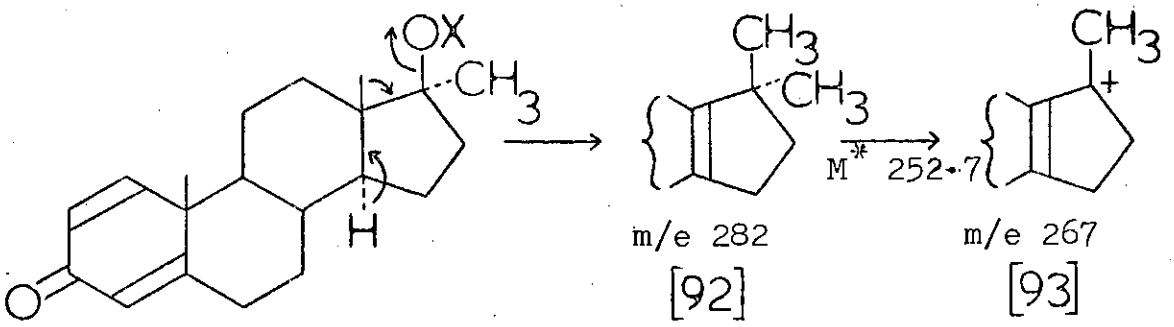


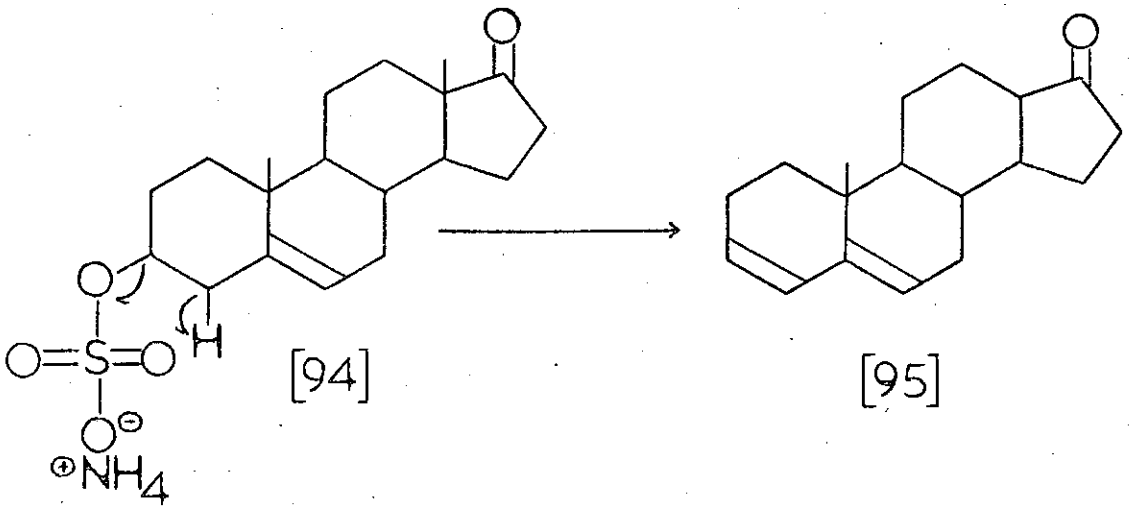
Figure 12. The mass spectra of methandrostenolone triethylammonium sulphate a) at 100° and b) at 200°.

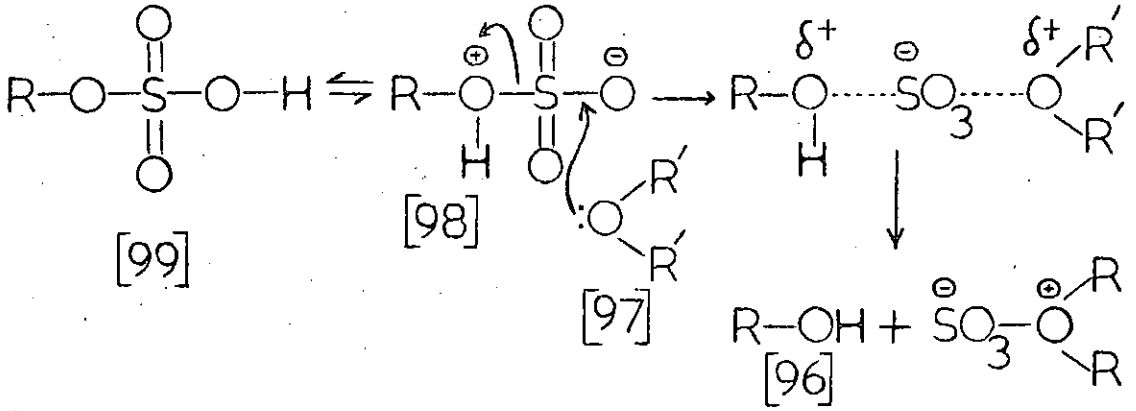
spectrometry of steroid sulphates, probably because the temperatures required to obtain adequate signal response lead to decomposition of the salts presently used and hence the method is of limited value for the characterisation of these compounds.[147b] Joseph and Dusza have subjected a number of triethylammonium sulphates to mass spectrometry, the detailed results of which are not available.[147b] However, ions at m/e 48, m/e 64 and m/e 101, indicative of the SO^+, SO_2^+ and $(C_2H_5)_3N^+$ fragments, respectively, were found although no molecular ions were observed.[147b] The spectrum at 100° of methandrostenolone triethylammonium sulphate [figure 12a] prepared by the method described by Roy and Trudinger[159] was similar to those of all the samples studied [see figure 11a] and showed no evidence of the three above-mentioned ions. In the spectrum of the same sample at 200° [figure 12b] the high mass fragments had declined and among the prominent peaks were those at m/e 48, m/e 64 and m/e 101. Joseph *et al.*[161] reported that the base peak in the mass spectrum of 3-hydroxy-2-methoxyoestra-1,3,5(10)-trien-17-one potassium sulphate was the molecular ion of the free steroid, but that the SO^+ and SO_2^+ ions were not observed because the temperature was insufficient to cause decomposition of the potassium salt obtained by cleavage of the S-O bond.

The results described thus indicated that methandrostenolone pyridinium[90] and triethylammonium[91] sulphates were successfully formed and, inside the mass spectrometer, decomposed to give a species with intense ions at m/e 282[92] and m/e 267[93][see figure 11a]. The metastable ion at m/e


OSO3HNC5H5
CH3
OH
O
CH3
[90]
OSO3HN(C2H5)3
CH3
[91]


OX
CH3
H
CH3
CH3
M* 252·7
CH3
+
m/e 282
m/e 267
[92]
[93]


O
O=S=O
O⊖
⊕NH4
H
[94]
[95]


R–O–S–O–H
O
O
[99]
R–O–S–O
H
O
O
[98]
R′
O
R′
[97]
δ+
δ+
R′
R′
R–O
H
SO3
O
R–OH +
SO3–O
R
R
[96]

252.7 showed that both ions originated from the same molecule. The low intensity ion at m/e 300 may have arisen from cleavage of the S-O bond in the spectrometer,[161] from unreacted methandrostenolone or from derivative hydrolysed prior to spectrometry. A low intensity ion at m/e 239, which occurred in neither the spectrum of methandrostenolone nor that of its 17-epimer, and the almost total disappearance of the ion at m/e 242 indicated that the sulphate decomposed in the mass spectrometer to give the 18-nor-17,17-dimethyl-$\Delta^{13(14)}$-ene[92] (M^+, m/e 282). The mass spectrum [figure 11b] of the major product of the sulphuric acid catalysed elimination reaction of methandrostenolone,17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one[92] was similar to those produced by the sulphates of methandrostenolone [see figure 11a]. Elimination of the sulphate to the 17-methyl-Δ^{16}- molecule would give rise to a species with M^+ at m/e 282. However this species contributes to the m/e 282 ion in the spectra of methandrostenolone and its 17-epimer, neither of which contains the ion at m/e 239. In addition, exocyclic elimination would be expected to accompany endocyclic elimination of the sulphate in which event the m/e 267 ion would not be expected to be as intense as observed [see figures 11a and 12a]. Joseph et al.[161] found that the mass spectrum of 3β-hydroxyandrost-5-en-17-one ammonium sulphate[94] contained no molecular ion but that the base peak arose from elimination of the elements of ammonium sulphate to give a $\Delta^{3,5}$-diene ion[95].

The hydrolysis of steroid hydrogen sulphates has been the subject of much study[148,175] because of the importance of

establishing whether or not the steroids identified after the hydrolysis of sulphates of biological origin are the products of rearrangement and hence only artefacts of the hydrolysis procedure. The solvolysis reaction can be expressed as nucleophilic displacement of the alcohol (R-OH) [96] by the ether (R'_2O)[97] from the reactive dipolar species [98] which is regarded as existing in equilibrium with the other tautomeric forms of the hydrogen sulphate [99]. The effectiveness of various ethers in accomplishing the solvolysis follows their order of basicity.[117b] Because of the low basicity of the phenolic oxygen atom sulphates of the oestrogens are so readily hydrolysed that they are difficult to handle.[117b] Prediction of the basicity of the C-17 oxygen atom of methandrostenolone is difficult but hydrolysis of methandrostenolone sulphate must have occurred in carbon disulphide, used for infrared spectroscopy, in acetone prior to t.l.c. and perhaps partially in deuteriochloroform during n.m.r. spectroscopy. This mode of hydrolysis, i.e. fission of the O-S bond, also occurred when the sulphate was refluxed in an aqueous basic medium, giving methandrostenolone as the product.

3.9.v. The attempted synthesis of methandrostenolone sodium sulphate

A mild method of sulphation employing dicyclohexylcarbodiimide to generate the reactive species from sulphuric acid has been described by Mumma *et al.*[166] The synthesis of the sodium salt of methandrostenolone sulphate was attempted by this method and a product was obtained in very low yield

after passage through two ion-exchange columns. The positions of the methyl peaks in the n.m.r. spectrum of the product differed from those of methandrostenolone but resembled those of the products of dehydration of the 17β-hydroxyl group (see section 3.13). The nature of the product was confirmed by its low polarity on a t.l.c. plate; thus the steroid was dehydrated by sulphuric acid rather than sulphated. The low recovery of product (ca 20%) was then explained by the fact that the first ion-exchange column was designed to remove any unreacted steroid from the sulphate, the former being eluted first and discarded.

3.9.vi. The synthesis of the sulphates of dehydroepiandrosterone, testosterone, methyltestosterone and 17α-hydroxyprogesterone

Dusza et al.[164] reported details of the n.m.r. spectra of a variety of triethylammonium sulphates including those of dehydroepiandrosterone, testosterone and 17α-hydroxyprogesterone. These compounds and the derivative of methyltestosterone were prepared and the n.m.r. spectra recorded. All contained a quartet and a triplet in the correct positions for the sulphates, although the peaks may have been intensified by chemical exchange of the ammonium proton with free base. The spectra of the derivatives of dehydroepiandrosterone and testosterone contained peaks at the positions reported[164] for the sulphates and at those of the free steroids whereas those of 17α-hydroxyprogesterone and methyltestosterone, like methandrostenolone, showed no shifts from the positions of the peaks in the free steroid.

Dehydroepiandrosterone pyridinium sulphate was prepared, the signal due to the 3α-proton being shifted downfield in the n.m.r. spectrum of the derivative compared to that of the free steroid. These results confirmed that secondary sulphates are more easily prepared and of greater stability than the derivatives of tertiary alcohols.

3.9.vii. The synthesis of sulphonates of methandrostenolone

The nucleophilic substitution of sulphonic esters of secondary steroidal alcohols, particularly the toluene-p-sulphonates, has been extensively studied for mechanistic purposes and as a means to the epimerisation of alcohols.[117c] Derivatives of tertiary alcohols have not been so studied; nevertheless the preparation of methandrostenolone tosylate was attempted. Recovery of the derivative by precipitation by the addition of water to the reaction mixture proved unsatisfactory and so the solvent was removed by evaporation at room temperature and the crystalline product obtained by filtration. The tosylation product behaved similarly to the sulphates described previously; due to rapid hydrolysis of the tosylate infrared spectroscopy and t.l.c. indicated that the reaction had not occurred but the mass spectrum was similar to those given by the sulphate derivatives [see figure 11a].

Nucleophilic substitution is generally accompanied by elimination, the ratio of products from each mechanism varying according to the reaction conditions.[117c] Accordingly the product of tosylation of methandrostenolone was adsorbed onto

alumina. Although alumina tends to operate as a base rather than a nucleophile[117c] and thus to cause predominant elimination only 5% of the product was of sufficiently low polarity to be eluted with benzene. The remaining 95% of the material on the column was eluted as a single fraction with similar polarity and identical mass spectrum to methandrostenolone. The trimethylsilyl ether of this fraction was prepared and examined by g.l.c. but no trace of 17-epimethandrostenolone was detected. When authentic androstanolone tosylate was prepared and adsorbed onto alumina no elimination or inversion products were detected. The major product was unchanged derivative and the other product was the free 17β-hydroxy steroid.

The methanesulphonate of methandrostenolone was synthesised and found to be insoluble in common solvents, such as those used for infrared and n.m.r. spectroscopy. The mass spectrum of the derivative was similar to those of the sulphates and tosylate of methandrostenolone [see figure 11a]. After hydrolysis of the mesylate, methandrostenolone was obtained.

3.9.viii. Conclusion

The results described in this section indicated that it was possible to prepare the sulphate and sulphonate derivatives of the tertiary hydroxyl group of methandrostenolone but that, as expected, these derivatives were unstable[168] and decomposed to give the parent steroid in common solvents and when intentionally hydrolysed. Thus, chemical hydrolysis resulted in fission of the S-O bond. The mass spectral results

were, however best explained by fission of the C(17)-O bond in the mass spectrometer, with migration of the C-18 methyl group to C-17 and elimination of the 14α-hydrogen atom to give the quaternary-substituted $\Delta^{13(14)}$-double bond. Inversion at the 17-position would require fission of the C(17)-O bond and attack by a hydroxyl group from the α-side of the molecule before migration of the C-18 methyl group could occur. It is feasible that an enzyme, by virtue of its stereochemistry, might prevent C-18 methyl migration and thus allow inversion to proceed.

3.10. Experimental Section

3.10.i. Pyridine-sulphur trioxide[159]

A mixture of chlorosulphonic acid (4ml) and ethanol-free chloroform (20ml) was added dropwise to a mixture of dry pyridine (10ml) and chloroform (30ml) at 0°C. The reaction vessel was surrounded by an ice-salt bath and the rate of addition adjusted such that the temperature remained below 5° during the addition, which required approximately 2 hours. The white precipitate of pyridine-sulphur trioxide was recovered by filtration, washed with cold chloroform (3 x 8ml) to remove pyridine hydrochloride and with water (2 x 8ml). The product was dried over phosphorus pentoxide to give pyridine-sulphur trioxide (7.5g, 76%); m.p. 140° (lit.[163] 97-175°).

3.10.ii. Methandrostenolone triethylammonium sulphate

a. Room temperature for 2 hours

Pyridine-sulphur trioxide complex (26.9mg, Aldrich Chemical Co. Inc., Milwaukee, Wis. U.S.) and triethylamine (0.23ml) were added to a solution of methandrostenolone (46.0mg) in a small volume of dry pyridine and the reaction flask stoppered and left at room temperature for 2 hours. Anhydrous ether (ca. 10 times the volume of the reaction mixture) was added, whereupon a cloudy white precipitate, which could not be filtered, was observed. The solvents were removed by evaporation with a gentle stream of nitrogen at room temperature and crystallisation occurred after storage in a desiccator in vacuo. The pale yellow crystals were filtered under suction and washed with anhydrous ether.

Infrared spectra of the crystals (m.p. 142-147°) and of the ether-soluble material were identical with that of methandrostenolone. Similarly, t.l.c. of these fractions and the crude product showed only the free steroid. The n.m.r. spectrum (100 M Hz) of the crystals was similar to that of methandrostenolone but for an additional quartet and triplet; τ9.08 (C-18 methyl), 8.82 (C-20 methyl), 8.76 (C-19 methyl), 8.65 (t, 7 Hz), 6.84 (q, 7 Hz), [triethylamine, τ9.03 (t, 7.2 Hz), 7.57 (q, 7.2 Hz); diethyl ether, τ8.81 (t, 7.2 Hz), 6.52 (q, 7.2 Hz); triethylammonium sulphates,[164] τ8.67 ± 0.08 (t), 6.92 ± 0.08 (q)]. The ethyl group was present in ca. 20% of the sample. Mass spectra of the crystals (the intensities [% base peak] in brackets refer to spectra at 120° [figure 11a], 125° and 140°, respectively); m/e 300 (6,7,5%), m/e 282 (49,100,100%), m/e 267(27,53,73%), m/e 239 (3,4,5%).

b. 60-70° and then at room temperature for 48 hours[159]

Methandrostenolone (51.8mg) and pyridine-sulphur trioxide (preparation described in section 3.10.i, 50mg) were suspended in dry pyridine (0.25ml) and warmed to 60-70° to give a pale yellow solution above clear droplets. The mixture was allowed to cool whereupon the droplets crystallised. Triethylamine (2 drops) was added and after 48 hours at room temperature a single-phase pale yellow solution was obtained. The solvents were evaporated at room temperature with nitrogen and the last traces removed in a desiccator in vacuo to yield a yellowish-green product (73.1mg); m.p. 143-150°; infrared spectrum and t.l.c. similar to methandrostenolone; mass spectrum (intensities at 115 and 130°);

m/e 300 (5,3%), m/e 282 (57,45%), m/e 267 (26,33%), m/e 239 (3,3%); calculated for $C_{26}H_{43}O_5SN$, C = 64.84, H = 9.00, N = 2.91%, found, C = 62.02, H = 8.78, N = 2.53% (calculated for methandrostenolone, C = 80.00, H = 9.34%).

c. 80-85° for 4 hours[164]

Pyridine-sulphur trioxide (29mg, 1.1 equivalents) and triethylamine (4 drops) were added to a solution of methandrostenolone (50mg) in a small volume of pyridine. The reaction vessel was fitted with a reflux condenser protected with a calcium chloride tube and heated at 80-85° for 4 hours. The orange solution was allowed to cool before the addition of anhydrous ether (ca. 10-20 times the volume of the reaction mixture). A white precipitate, which could not be filtered, was obtained. It was thus dissolved by the addition of methylene chloride and the solvents were removed at room temperature by evaporation in vacuo, by a stream of nitrogen and finally in a desiccator in vacuo. The infrared spectrum of the crystalline product (m.p. 144-154°) was similar to that of methandrostenolone, as was the n.m.r. spectrum (100 M Hz) but for ethyl protons at τ8.67 (t, 7 Hz, 9 H), 6.83 (q, 7 Hz, 6H). T.l.c. of the product in the system benzene, acetone, water (2:1:2), upper phase, 70%-methanol, 30%[164] showed two similarly coloured spots at R_f 0.86 (major), corresponding to methandrostenolone and at R_f (0.5).

3.10.iii Hydrolysis of methandrostenolone triethylammonium sulphate

Methandrostenolone triethylammonium sulphate (52.5mg, preparation described in section 3.10.ii.b.), sodium bicarb-

onate (ca. 1mg) and water (2ml) were heated at 100°C for 1 hour. After cooling, the steroid was extracted into ether and the ether extract washed with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a crude product (36.2mg); m.p. 153-157°; n.m.r. (100 M Hz) τ9.08 (C-18 methyl), 8.82 (C-20 methyl), 8.77 (C-19 methyl), 3.94 (d, 2 Hz, 4-H), 3.79 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.94 (d, 10 Hz, 1-H); t.l.c., polarity and colour reaction identical to those of methandrostenolone; mass spectrum, m/e 300 (17%), m/e 282 (11%), m/e 267 (7%), m/e 242 (16%).

3.10.iv. Methandrostenolone pyridinium sulphate

Methandrostenolone (48.8mg) and pyridine-sulphur trioxide (50mg) were suspended in pyridine (0.25ml) and heated to give a solution. The reaction mixture was then left at room temperature for 48 hours. Yellowish-green crystals formed and were recovered by filtration and dried in a desiccator in vacuo. Yield 62.9mg; m.p. 122-130°; Mass spectrum, 115°, m/e 282 (100%), m/e 267 (70%), m/e 239 (4%).

3.10.v. Attempted synthesis of methandrostenolone sodium sulphate[166]

Methandrostenolone (50mg) was dissolved in dimethylformamide (0.2ml) and added to an ice-cold solution of dicyclohexylcarbodiimide (0.17g) in dimethylformamide (0.3ml). Concentrated sulphuric acid (0.014ml) in dimethylformamide (0.1ml) was added and the mixture kept at 0° for 15 minutes, with occasional agitation. It was then transferred to a column of DEAE cellulose (Whatman DE-11, Cl^- form, 4g) and

methanol (1 litre) was passed through the column to elute unreacted steroid and dicyclohexylcarbodiimide, dimethylformamide and dicyclohexylurea. This fraction was discarded. 1M methanolic ammonium hydroxide (200ml) was passed through the column and the solvents removed from this fraction by evaporation below 40° *in vacuo*. The residue was suspended in acetone (100ml) and the white precipitate of ammonium chloride removed by filtration. The filtrate was passed through a column of Dowex-50W-X8 (Na^+ form) and the eluent removed by evaporation below 40°. The residue was dried over phosphorus pentoxide to give a brown glass (*ca.* 10mg); n.m.r. (100 M Hz) showed that the D-ring differed from that of methandrostenolone; t.l.c. in chloroform-methanol-water (65:25:4)[166] showed that the product had similar polarity to methandrostenolone (R_f *ca.* 0.88) and in benzene-ethanol (9:1) two components were resolved, one similar to methandrostenolone and one less polar.

3.10.vi. Dehydroepiandrosterone triethylammonium sulphate[164]

Dehydroepiandrosterone (250mg) and pyridine-sulphur trioxide (250mg) were suspended in pyridine (1.25ml) and warmed to 60-70° until complete solution was obtained. The reaction mixture was cooled, whereupon crystallisation occurred, and triethylamine (0.125ml) was added. After 48 hours at room temperature the solvents were removed with a stream of nitrogen and finally in a desiccator *in vacuo*. The infrared spectrum of the product was similar to that of the free steroid but t.l.c. showed two components, one with the same polarity as the free steroid and one which remained at the

start of the chromatogram; N.m.r. (60 M Hz) τ9.14 (C-18 methyl), 8.98 (C-19 methyl), 8.64 (t, 7 Hz), 6.84 (q, 7 Hz), 5.74 (broad, 3α-H), 4.61 (m, 6-H) dehydroepiandrosterone triethylammonium sulphate [lit.,[164] τ9.18, 8.98, 5.75, 4.60 and free dehydroepiandrosterone, τ9.11, 8.96, 6.52, 4.62].

3.10.vii. Testosterone triethylammonium sulphate[164]

Testosterone (250mg) was reacted with pyridine-sulphur trioxide (250mg) and triethylamine (0.125ml) in pyridine (1.25ml) as described in section 3.10.vi. The infrared spectrum of the product was similar to that of testosterone, but t.l.c. showed a component remaining at the start of the chromatogram as well as one corresponding to testosterone. N.m.r. (60 M Hz) τ9.17 (C-18 methyl), 8.83 (C-19 methyl), 8.64 (t, 7 Hz), 6.84 (q, 7 Hz), 5.71 (17α-H), 4.30 (4-H) testosterone triethylammonium sulphate [lit.,[164] 9.18, 8.80, 5.63, 4.25] and τ9.23 (C-18 methyl), 8.83 (C-19 methyl), 6.35 (17α-H), 4.30 (4-H) testosterone [τ9.21, 8.82, 6.35, 4.23].

3.10.viii. Methyltestosterone triethylammonium sulphate[164]

Pyridine-sulphur trioxide (57mg) and triethylamine (8 drops) were added to a solution of methyltestosterone (100 mg) in a small volume of dry pyridine. The reaction vessel was fitted with a reflux condenser protected with a calcium chloride tube and heated at 80-85° for 4 hours. The orange solution was allowed to cool and the solvent removed by evaporation in vacuo below 40°, then with a stream of nitrogen and finally in a desiccator in vacuo, whereupon crystallisation occurred. The product was examined by n.m.r.

spectroscopy (100 M Hz); τ9.10 (C-18 methyl), 8.80 (C-19 methyl), 8.80 (C-20 methyl), 8.66 (t, 7 Hz), 6.80 (q, 7 Hz), 5.40 (broad, N H), 4.28 (4-H) methyltestosterone triethylammonium sulphate [methyltestosterone, τ9.09, 8.79, 8.79, 4.27].

3.10.ix. 17α-Hydroxyprogesterone triethylammonium sulphate[164]

17α-Hydroxyprogesterone (100mg) was reacted with pyridine-sulphur trioxide (57mg) and triethylamine (8 drops) in pyridine as described in section 3.10.viii. The product was examined by n.m.r. spectroscopy (100 M Hz); τ9.28 (C-18 methyl), 8.82 (C-19 methyl), 8.67 (t, 7 Hz), 7.75 (C-21 methyl), 6.80 (q, 7 Hz), 4.27 (4-H), 4.01 (broad, N H) 17α-hydroxyprogesterone triethylammonium sulphate [lit.,[164] 9.35, 8.84, 7.72, 4.30].

3.10.x. Methandrostenolone tosylate

Methandrostenolone (50mg) was dissolved in dry pyridine (7ml), cooled to 0° and added to a solution of freshly purified toluene-p-sulphonyl chloride[112c] (67mg) in pyridine (3.5ml) at 0°. The reaction mixture was kept at 0° for 4 days after which time it was a faint pinkish-yellow colour. Pyridine was removed by evaporation at room temperature with nitrogen and the solid material was washed with pyridine and dried by filtration and storage in a desiccator in vacuo. Infrared spectroscopy and t.l.c. of the pale yellow crystals (60mg) indicated only unreacted methandrostenolone. However the mass spectrum of the crystals was different from that of the free steroid; 140°, m/e 282 (77%) m/e 267 (60%), m/e 239 (12%).

3.10.xi. Reaction of methandrostenolone tosylate with alumina

Methandrostenolone tosylate (200mg) was adsorbed onto alumina (5g) and benzene (100ml) was passed through the column, which was then left for 3 days. Non-crystalline material (5.3mg) was eluted with benzene. This fraction was of low polarity compared with methandrostenolone and probably contained one or more products of elimination at C-17; it was not examined further. A yellow glass (45mg), which crystallised on standing, was eluted with benzene-chloroform (1:1, 100ml). The polarity and mass spectrum indicated that it was methandrostenolone; m/e 300 (31%), m/e 282 (11%), m/e 267 (7%), m/e 242 (17%); G.l.c. of the trimethylsilyl ether of this fraction showed no trace of 17-epimethandrostenolone.

3.10.xii. Androstanolone tosylate

An ice-cold solution of androstanolone (5.0g) in pyridine (70ml) was added to a solution of freshly purified toluene-p-sulphonyl chloride[112c] (6.0g) in pyridine (30ml) at 0°. The pale yellow solution was maintained at 0° for 48 hours by which time a bright orange-pink colour had developed. Water was added and the precipitate recovered by filtration, washed with water, dried and recrystallised from ethanol to yield androstanolone tosylate (5.99g, 78%); m.p. 175.5-177°; ν_{max} 1708,1380,1193,1183cm^{-1}; n.m.r. (100 M Hz) τ9.20 (C-18 methyl),9.02 (C-19 methyl), 7.56 (Ar-C$\underline{H}_3$), 5.74 (t, 8 Hz, 17α-$\underline{H}$), 2.69, 2.23 (d,d,9 Hz, 9 Hz, 4 H, Ar $\underline{H}$).

3.10.xiii. Reaction of androstanolone tosylate with alumina

Androstanolone tosylate (3.125g) was dissolved in a small volume of benzene and adsorbed onto alumina (200g). After 48 hours the column was eluted with petrol and with benzene, both of which removed a single compound (1.630g) which, from its polarity, infrared and n.m.r. spectra was unchanged androstanolone tosylate. Elution with ethanol gave a mixture (0.375g) of androstanolone and androstanolone tosylate (2:1); n.m.r. (60 M Hz) τ9.23 (C-18 methyl), 8.96 (C-19 methyl), 6.34 (t, 7 Hz, 17α-H) androstanolone and τ9.20 (C-18 methyl), 9.00 (C-19 methyl), 7.55 (Ar-CH_3), 5.73 (t, 8 Hz, 17α-H), 2.65, 2.20 (d,d,9 Hz, 9 Hz, Ar-H) androstanolone tosylate.

3.10.xiv. Methandrostenolone mesylate

Ice-cold methanesulphonyl chloride (0.04ml) was added to a solution of methandrostenolone (51.5mg) in dry pyridine (0.5ml) at 0°. A pale yellow colour developed and after 18 hours at room temperature colourless crystals had formed. The white crystalline product (40.2mg) was recovered from the dark yellow mother liquor by filtration. The crystals (m.p. 154-158°) were too insoluble in carbon disulphide and chloroform to obtain infrared and n.m.r. spectra, respectively. The mass spectrum of the crystals, 140°, m/e 282 (45%), m/e 267 (26%), m/e 239 (2%) showed that they were not methandrostenolone.

3.10.xv. Hydrolysis of methandrostenolone mesylate

The product of the preceding reaction (15.7mg) was

suspended in water (1ml) with a trace of sodium bicarbonate and heated at 100° for 1 hour. The steroid was extracted into ether, washed with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a white solid (3.3mg); m.p. 150-154°; polarity and mass spectrum similar to those of methandrostenolone; 130°, m/e 300 (27%), m/e 282 (24%), m/e 267 (9%), m/e 242 (19%).

3.11. The synthesis of the $\Delta^{1,4}$-dien-3-one system from Δ^{4}-ene-3-keto and Δ^{4}-ene-3β-hydroxy precursors

The original source of methandrostenolone used for this work was extracted from commercial tablets. However, the synthesis of methandrostenolone from the readily available precursor, methyltestosterone, was examined as a more accessible source of the compound. Industrially the Δ^{1}-double bond is introduced into the precursor, usually methyltestosterone, chemically or microbiologically.[5c] The methods of 1-dehydrogenation are well established but difficulty arises in the separation of the product from methyltestosterone in the event of incomplete reaction. The variety of synthetic modifications and separation methods described in the literature[176-180] confirm that this is an important industrial problem in the synthesis of methandrostenolone. The androgenic nature of methyltestosterone makes its presence intolerable in a drug which may be administered to women and children.

Among the methods which have been used to reduce the interference by methyltestosterone is an enzymic dehydrogenation in which the substrate was reported to be preferentially absorbed and retained by the cells whilst the product was slowly released into the liquid phase which was periodically removed.[176] Adduct formation between steroidal Δ^{4}-en-3-ones, and 2-naphthol[177] and morpholinohydrazide derivatives[178] and the different rates of hydrolysis of the semicarbazones[179] of Δ^{4}-en-3-ones and $\Delta^{1,4}$-dien-3-ones have all been used as means of purification. The microbial oxidation and dehydrogenation of 17α-methylandrost-4-ene-3β,17-diol has been

described as a means of avoiding the complications caused by the presence of methyltestosterone.[180]

The problem of the purification of methandrostenolone from methyltestosterone was identical to that encountered in the synthesis of 17-epimethandrostenolone, since each pair of epimers have similar polarities. Purification was achieved by column chromatography and recrystallisation in the synthesis of the latter compound. This method produced sufficient pure material for the characterisation of the metabolite of methandrostenolone but would be inefficient for the preparation of large quantities of the dienone.

The use of 3-4 equivalents of DDQ has been recommended for the conversion of a saturated 3-hydroxysteroid to the corresponding $\Delta^{1,4}$-dien-3-one.[119] Consequently, for the preparation of the dienone from a steroid already partially dehydrogenated in the A-ring proportionally less reagent was used. However, reaction of methyltestosterone with 1.1 equivalents of DDQ gave only slightly more than 50% of the dienone. Preparative t.l.c. was inefficient for purification of the product because the polarities of the compounds are so similar that overlapping bands are obtained. G.l.c. showed that repeated crystallisation resulted in enrichment of the filtrate in methandrostenolone. Dichlorodicyanobenzoquinol was readily removed from the product by filtration, but unreacted DDQ was difficult to remove. Filtration through alumina resulted in the adsorption of steroids and reagent but the method described by Caspi *et al.*,[181] which employed a wash with sodium hydroxide solution, removed all DDQ.

In view of the poor conversion to dienone and the inefficiency of the conventional methods of purification other separation methods were examined. As mentioned previously, semicarbazone derivatives[179] and 2-naphthol adducts[177] have been utilised. A Japanese group has described the use of morpholinoacetohydrazide and 4-hydrazinoformylmethyl-4-methyl-morpholinium halides for the separation of methandrostenolone from methyltestosterone.[178] They reported that the latter compounds formed water-soluble derivatives with the 4-en-3-one, but did not react with the dienone, thereby making separation of the two compounds easy.

4-Hydrazinoformylmethyl-4-methylmorpholinium chloride was prepared, as nearly as possible, according to the method of Tohda et al.[178] Crystallisation could not be accomplished although it was obvious from the melting point reported[178] for the compound that the crystalline form was attainable. The viscous oily product was assumed to be the desired reagent, and its reactions with a Δ^4-en-3-one(testosterone) and a $\Delta^{1,4}$-dien-3-one(cholesta-1,4-dien-3-one) were examined prior to an attempted separation of methandrostenolone and methyltestosterone. When the reagent was reacted with testosterone only 6.5% of the steroid was recovered from the organic phase whereas 81.3% of a sample of cholesta-1,4-dien-3-one was recovered from this phase. A mixture of methandrostenolone and methyltestosterone was then reacted with 4-hydrazinoformylmethyl-4-methylmorpholinium chloride and the organic extract was found to be free from the Δ^4-en-3-one.

Once a successful method for the separation of $\Delta^{1,4}$-dien-

3-one from unreacted Δ^4-en-3-one was available the synthesis of 17-epimethandrostenolone was reexamined. Theoretically, 2 equivalents of DDQ should be required to oxidise the 3-hydroxyl group and to introduce the Δ^1-double bond into 17β-methylandrost-4-ene-3β,17-diol. Consequently, as in the original synthesis of the compound (section 3.2.x.), 2.2 equivalents of DDQ were used. The progress of the reaction was monitored by periodic t.l.c. of the reaction mixture, which showed that oxidation to the 3-keto group occurred rapidly and completely within three hours of the start of refluxing. However, 1-dehydrogenation was gradual and incomplete even when the reaction was continued for twenty-four hours. Since Muller *et al.*[119] reported the conversion of a 3-hydroxysteroid to a $\Delta^{1,4}$-dien-3-one with 3-4 equivalents of DDQ, although in only 30% yield, the reaction of the Δ^4-ene-3β-hydroxy precursor of 17-epimethandrostenolone with 3.5 equivalents of DDQ was examined. After refluxing for fourteen hours, t.l.c. of the reaction mixture indicated complete conversion to the dienone. Consequently, 100% formation of the dienone was easily achieved provided that a large excess of DDQ was used, thus, obviating the necessity to use the tedious separation procedures described.

3.12. Experimental section

3.12.i. Reaction of methyltestosterone and 1.1 equivalents of DDQ[119]

DDQ (0.579g, 1.1 equivalents) was added to a solution of methyltestosterone (0.7g) in dry benzene (70ml) and the mixture was refluxed for 18 hours. The precipitated quinol was removed by filtration and washed with methylene chloride. The combined filtrate and washings were evaporated to dryness and the residue, a dark brown glass, was dissolved in methylene chloride-ether (1:3). The solution was filtered through a pad of alumina (10g), which was washed with ether. After evaporation of the solvents the residue was redissolved in the solvent mixture and the alumina filtration repeated. The filtrate was then washed[181] with 2N sodium hydroxide solution (30ml), which removed all colouration from the organic layer, and with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a pale yellow oil (0.66g). Crystallisation from acetone gave long white needles which were shown to be a mixture of slightly more methandrostenolone than methyltestosterone by t.l.c. G.l.c. of the crystals and residues after repeated crystallisations from acetone showed that the residue became richer in methandrostenolone. Preparative t.l.c. was attempted but overlapping bands were obtained. Methandrostenolone was more polar than methyltestosterone.

3.12.ii. 4-Hydrazinoformylmethyl-4-methylmorpholinium chloride[178]

4-Methylmorpholine (21.0g) in absolute ethanol (50ml) was mixed with ethylchloroacetate (19.9ml) and the solution

refluxed for 8 hours. As soon as the colourless solution was heated it rapidly changed through yellow and orange to deep red. The solution was allowed to cool and 100% hydrazine hydrate (11.2g) was added, whereupon most of the colour disappeared to give a pale orange solution from which a white viscous oily layer separated. This material was soluble in water but insoluble in ethanol but repeated attempts at crystallisation were unsuccessful. The supernatant was removed and benzene added and distilled to remove traces of water which might have hampered crystallisation. The final product was a creamy-white glass.

3.2.iii. Reaction of testosterone with 4-hydrazinoformylmethyl-4-methylmorpholinium chloride[178]

4-Hydrazinoformylmethyl-4-methylmorpholinium chloride (ca. 500mg) was added to a solution of testosterone (196.9mg) in a mixture of methanol and acetic acid (9:1, 8ml). The mixture was warmed to 40° and then left at room temperature for 2½ hours. Water and ether were added and the aqueous phase extracted twice with ether. The ether extracts were combined, washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a residue (12.8mg). T.l.c. showed that the residue contained testosterone and several more polar contaminants. Consequently, testosterone (184.1mg, 93.5%) was extracted into the aqueous phase by complex formation.

3.12.iv. Reaction of cholesta-1,4-dien-3-one with 4-hydrazinoformylmethyl-4-methylmorpholinium chloride[178]

A solution of cholesta-1,4-dien-3-one (55.6mg) in methanol and acetic acid (9:1, 2ml) was treated with the reagent (ca. 125mg) by the procedure described in the preceding section. Cholesta-1,4-dien-3-one (45.2mg, 81.3%) was recovered from the ether extracts.

3.12.v. Reaction of methandrostenolone and methyltestosterone with 4-hydrazinoformylmethyl-4-methylmorpholinium chloride[178]

A solution of a mixture (105.4mg) of methandrostenolone and methyltestosterone in methanol and acetic acid (9:1, 2ml) was treated with the reagent (ca. 250mg) as described in section 3.12.iii. A yellow glass (76.8mg, 72.8%) was recovered from the organic phase. T.l.c. showed that this product was pure methandrostenolone.

3.12.vi. Reaction of 17β-methylandrost-4-ene-3β,17-diol and DDQ[119]

a. 2.2 Equivalents of DDQ

DDQ (178.3mg, 2.2 equivalents) was added to a solution of 17β-methylandrost-4-ene-3β,17-diol (108.4mg) in redistilled dioxane (24ml) and refluxed. After 3 hours, t.l.c. of the reaction mixture showed complete conversion of the starting material to 17-methylepitestosterone, but no trace of 17-epimethandrostenolone. T.l.c. at intervals up to 24 hours after the start of the reaction showed gradual, but incomplete formation of the latter compound. The product was recovered as described in section 3.12.i. and contained a mixture of 17-methylepitestosterone and 17-epimethandrostenolone.

b. 3.5 Equivalents of DDQ

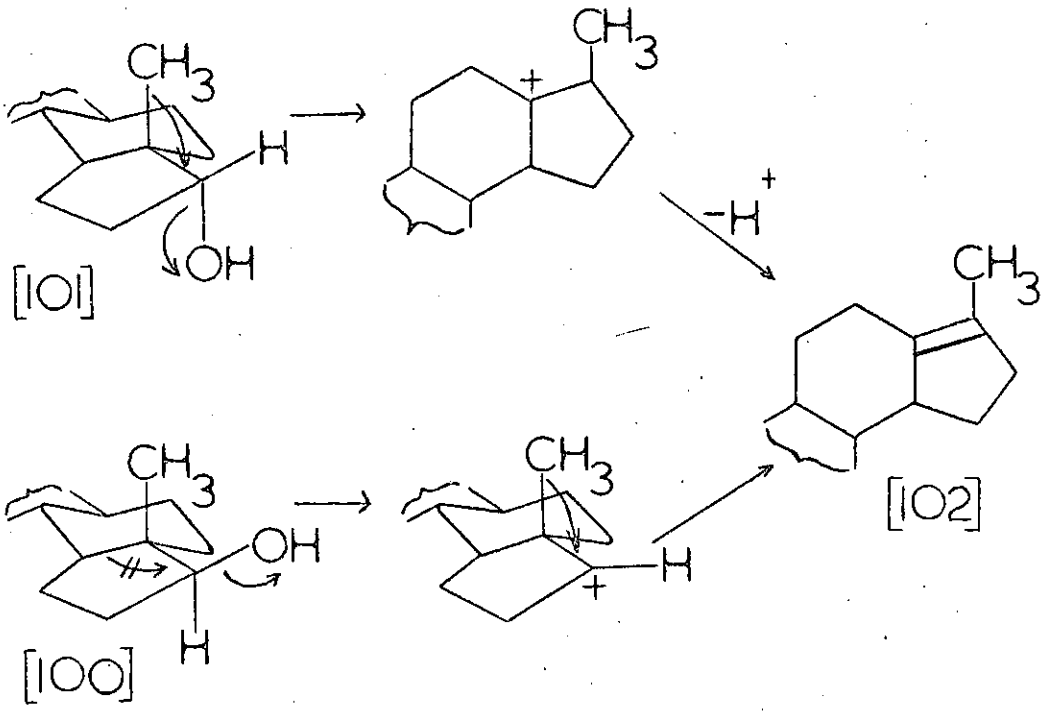
A solution of 17β-methylandrost-4-ene-3β,17-diol (475.3mg) and DDQ (1.250g, 3.5 equivalents) in dioxane (25ml) was refluxed for 14 hours, when t.l.c. of the reaction mixture showed complete conversion to 17-epimethandrostenolone. The product was recovered as described previously.

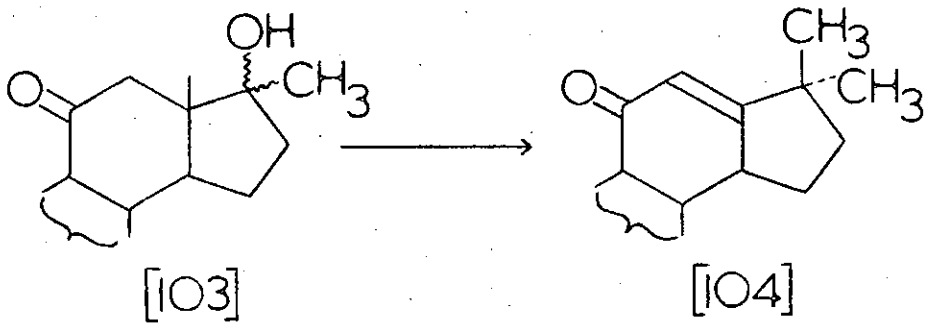
3.12.vii. Reaction of methyltestosterone and 3.1 equivalents of DDQ[119]

Methyltestosterone (5.034g) and DDQ (11.731g, 3.1 equivalents) were dissolved in dioxane (110ml) and refluxed for 6 hours when t.l.c. showed 100% reaction. The dioxane was removed by evaporation *in vacuo* and benzene was added to the residue, which was filtered to remove insoluble quinol. The quinol was washed with benzene and the filtrate and washings evaporated to dryness. The residue was dissolved in methylene chloride and ether (1:3), washed with 2N sodium hydroxide solution and then with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a crystalline product (4.668g, 93%). T.l.c. showed that this product was pure methandrostenolone and hence further purification was unnecessary.

3.12.viii. Purification of DDQ

Impure DDQ (40g, a mixture of quinol and quinone) was dissolved in benzene (400ml) and the insoluble quinol removed by filtration. The filtrate was evaporated to *ca.* 120ml and an equal volume of petrol ether (40-60°) was added to the hot solution. The bright red crystals of DDQ were removed by filtration and dried, below 40°, to give light orange crystals (28.5g); m.p. 212-214° [lit.,[112d] 213°].


CH3
H
OH
[101]
CH3
+
-H+
CH3
[102]
CH3
OH
H
[100]
CH3
H
+


OH
CH3
O
[103]
CH3
CH3
O
[104]

3.13. Some reactions of methandrostenolone

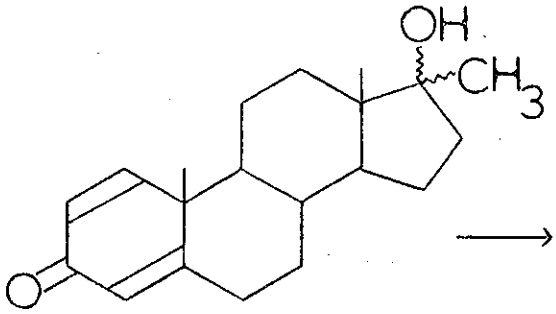
The reactions of methandrostenolone with a variety of reagents which would primarily cause reaction in the D-ring rather than the A-ring were examined. Reactions of the dienone system were not of interest in connection with the metabolism of methandrostenolone and have been studied extensively elsewhere[117d] because of the large number of compounds in which this system occurs.

3.13.i. Base

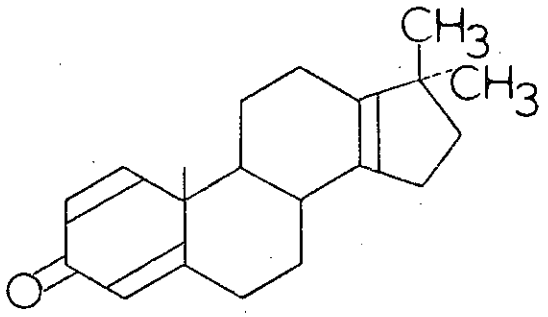
Methandrostenolone was refluxed with sodium methoxide and with sodium hydroxide in methanol. In both reactions only unchanged methandrostenolone was isolated.

3.13.ii. Acid

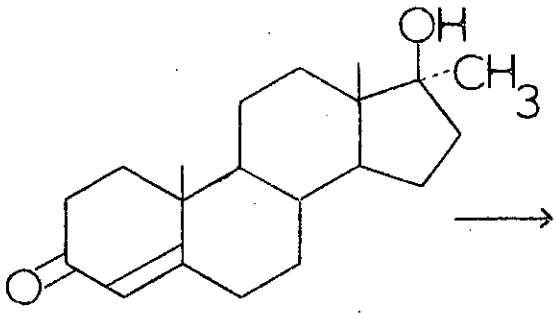
Secondary 17β-alcohols [100] are dehydrated slowly in comparison with the rate of the reaction of the corresponding 17α-ols[101], which have the trans-coplanar stereochemistry necessary for Wagner-Meerwein rearrangements.[117e] Migration of the 13,14-bond, which is trans-coplanar to the 17β-ol[100], is unfavourable because the product would contain a highly strained trans-fused cyclobutane system. Lack of anchimeric assistance thus means that formation of the 17-methyl-18-nor-$\Delta^{13(17)}$-compound[102] is slow. However, no difference has been observed in the rates of elimination of the two C-17 tertiary alcohols [103]; this has been ascribed to the increased stability of the C-17 tertiary carbonium ion compared to the corresponding secondary carbonium ion, an effect which must nullify any stereoelectronic difference.[117e,182a]



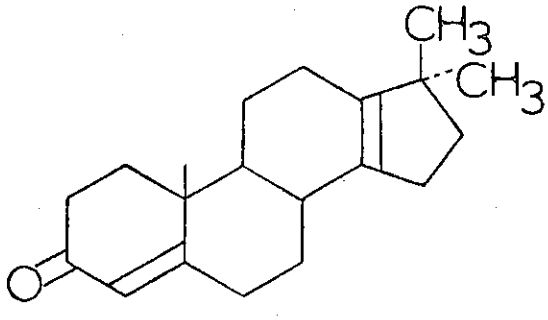
[105]



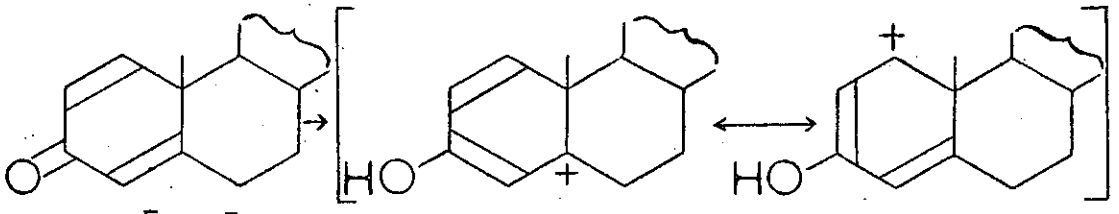
[106]



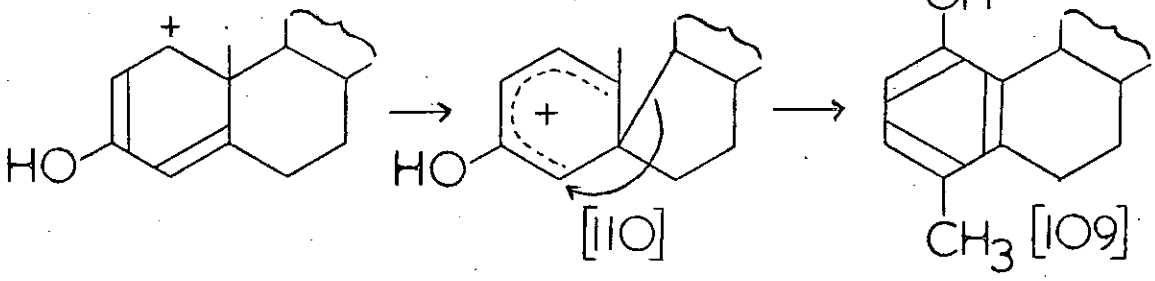
[51]



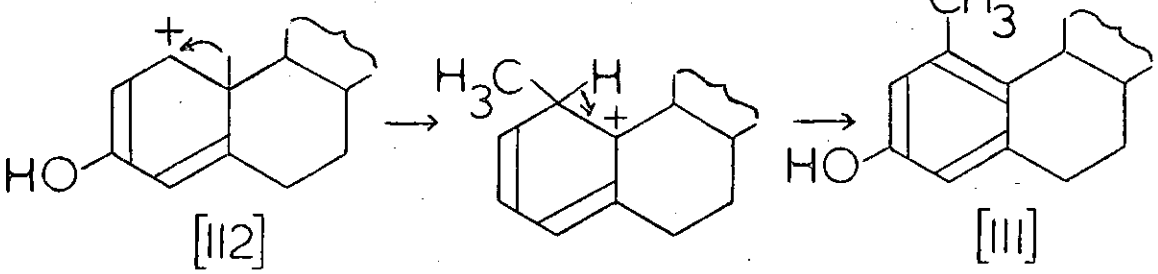
[107]



[108]



[110] [109]

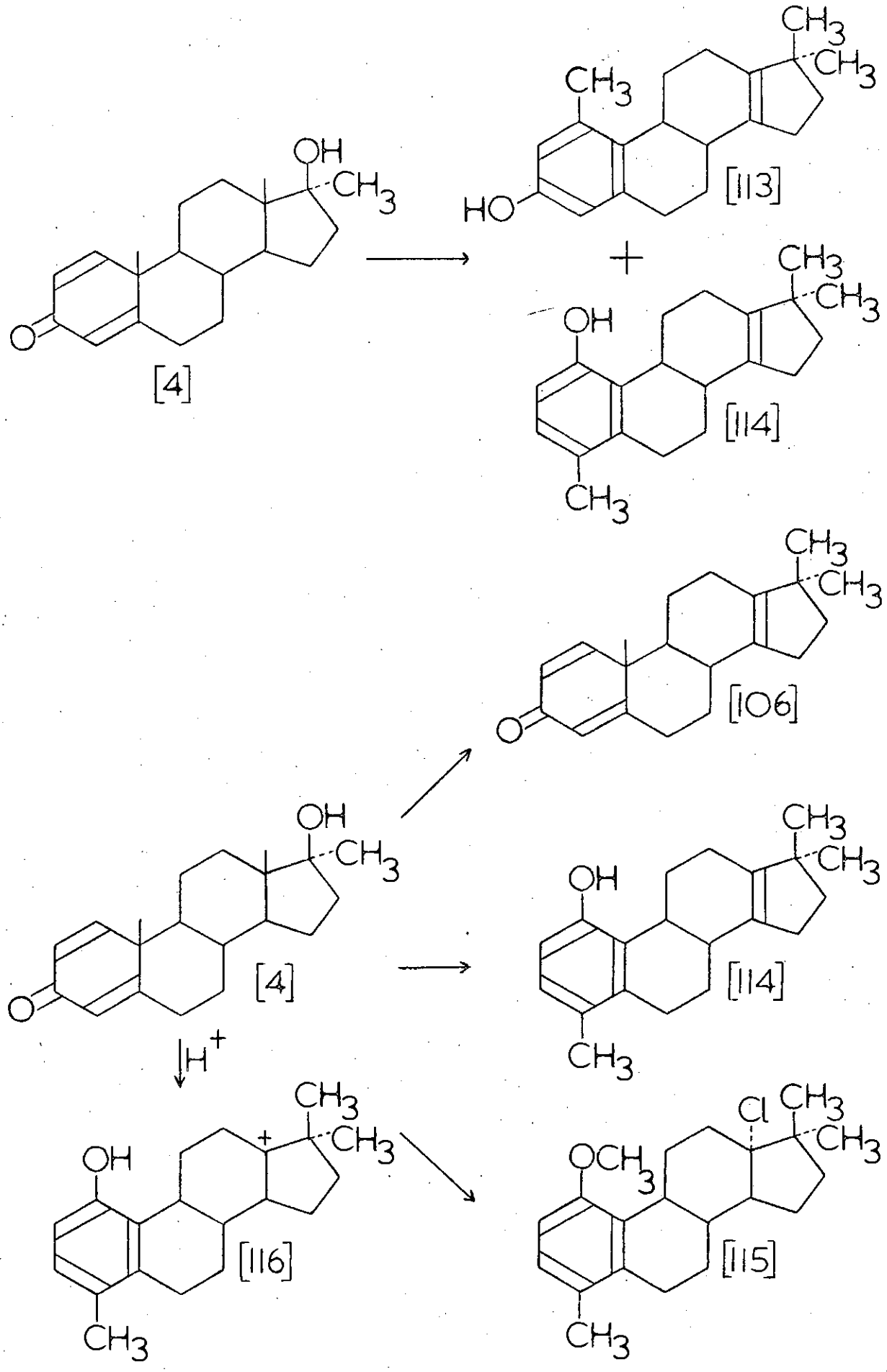


[112] [111]

Dehydration of a tertiary 17-alcohol may give products with a $\Delta^{13(14)}$- or a Δ^{12}-double bond [104], the latter being the major product from compounds with an 11-oxy substituent [103].

Methandrostenolone and its 17-epimer [105] were both heated with 20% aqueous sulphuric acid at 100° for 2 hours. Each compound reacted completely to give 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one[106] as the major product (ca. 50%), the absence of olefinic protons in the τ4.0-6.0 region of the n.m.r. spectra indicating that the extra double bond was tetrasubstituted. Methyltestosterone[51] reacted under similar conditions to give 17,17-dimethyl-18-norandrosta-4,13(14)-dien-3-one[107] as the only product.

The presence of a series of small peaks in the τ3.0-3.5 region of the n.m.r. spectrum of the product of the reaction of methandrostenolone with sulphuric acid indicated that the dienone-phenol rearrangement had occurred in about 50% of the sample. This reaction is normally carried out in acetic anhydride and sulphuric acid, when the major product[117d,182b] of a $\Delta^{1,4}$-dien-3-one[108] is the 1-hydroxy-4-methyl phenol[109]. The mechanism of the rearrangement under these conditions has been shown, with the aid of optically active compounds, to involve a spiran intermediate [110]. When the dienone contains certain substituents, such as a Δ^{6}-double bond, a C-6 or C-11 carbonyl group or a C-4 methyl group, all of which hinder the above mechanism, the major product[117d] is the 1-methyl-3-hydroxy phenol[111]. This compound probably arises by migration of the C-18 methyl group to a C-1 carbonium ion [112], followed by loss of the C-1 proton. In aqueous acids an unsubstituted $\Delta^{1,4}$-dien-3-


OH
CH3
O
[4]
CH3
CH3
CH3
HO
[113]
+
CH3
CH3
OH
[114]
CH3
CH3
[106]
O
OH
CH3
[4]
O
H+
OH
CH3
CH3
[114]
CH3
CH3
+
OH
[116]
CH3
Cl
CH3
CH3
OCH3
[115]
CH3

one was found to give predominantly the "meta" phenol [111], which may be largely due to the different polarity of the solvent.[117d] Consequently the product of the reaction of methandrostenolone [4] may have contained a mixture of the "meta" [113] and "para" [114] phenols, the former predominating.

When methandrostenolone was reacted with sulphuric acid in acetic acid the A-ring was completely rearranged, together with the expected D-ring reaction. Thus, although the D-ring rearrangement occurred when the steroid was not in solution (aqueous acid), solution was necessary for 100% dienone-phenol rearrangement (acetic acid). Under the latter conditions the major product was the "para" phenol [114], indicated by a pair of doublets at τ3.48, 3.15, J = 8 Hz in the n.m.r. spectrum. The coupling constants between meta protons and between para protons are 1-3 Hz and 0-1 Hz, respectively; thus the protons in the product were ortho (J = 6-9 Hz).[183]

Tishler and Brody[184] studied the reaction of methandrostenolone [4] with methanolic hydrochloric acid at 100° for 40 minutes and identified three of the products as 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one[106], 1-hydroxy-4,17,17-trimethyl-18-noroestra-1,3,5(10),13(14)-tetraene[114] and tentatively, 1-methoxy-4,17,17-trimethyl-13α-chlorooestra-1,3,5(10)-triene[115], the former being the major product. The latter two compounds fluoresced at 325 nm when activated at 280 nm which is characteristic of oestrogens, whereas the former compound exhibited no such fluorescence. All

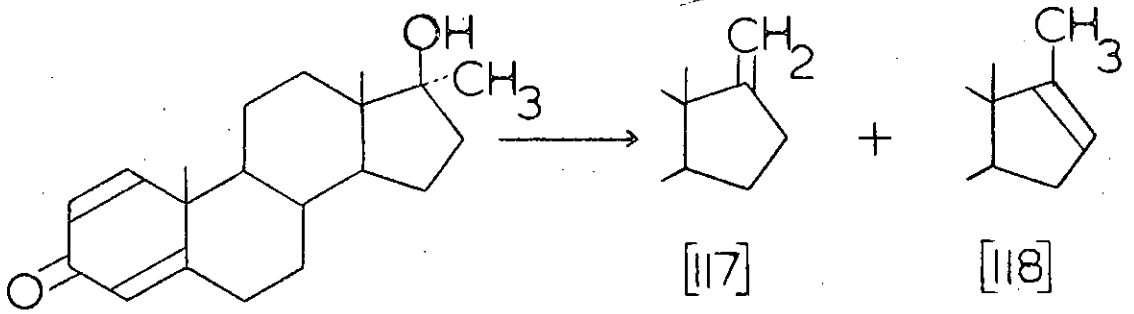
three compounds and methandrostenolone exhibited the same excitation and fluorescent spectra after heating with methanolic hydrochloric acid and it was suggested[184] that the C-13 carbonium ions of the two aromatic products were responsible for the fluorescent characteristics of methandrostenolone under these conditions.

The immediate generation of a coloured species was observed when methandrostenolone was heated with sulphuric and acetic acids; this may have been due to the C-13 carbonium ion [116].

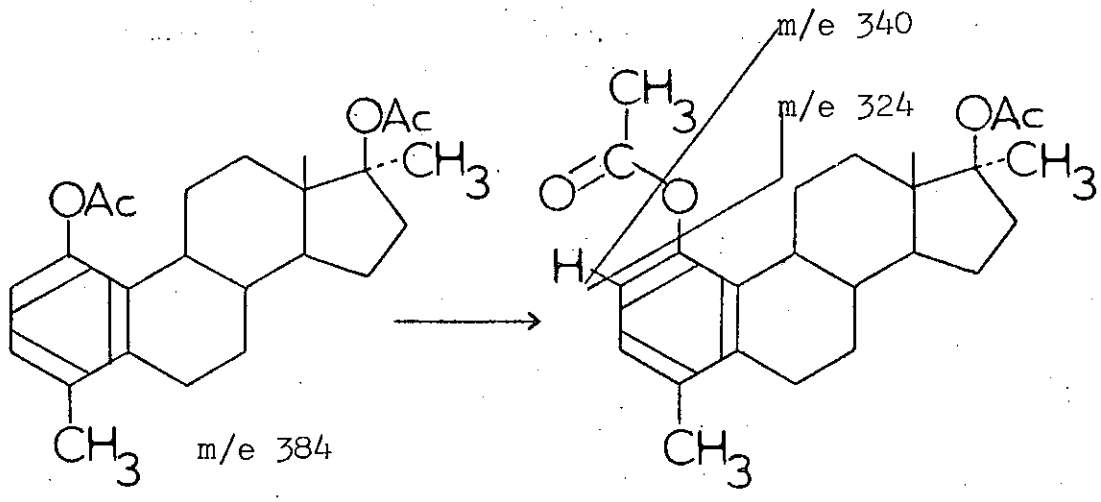
3.13.iii. Phosphorus oxychloride

The reaction of a steroid alcohol with phosphorus oxychloride or thionyl chloride has been shown to proceed stereospecifically through the trans-coplanar conformation of the leaving groups even when the product is the less stable olefin.[117f] In the case of a C-17 tertiary alcohol trans-coplanar elimination of the 17β-hydroxyl group with the 16α-proton requires distortion of the D-ring which is sterically hindered by the increased proximity of the 17β-substituent and the C-18 methyl group. Consequently exocyclic elimination is preferred.

In a study of 17a-methyl and 17a-ethyl-D-homo-17a-ols it was observed[89] that dehydration of the α-ols occurred readily in the mass spectrometer to give intense M-18 ions whereas the β-ols were chemically dehydrated with phosphorus oxychloride at a faster rate. This can be explained by the fact that the more crowded molecular ion (that in which the larger, alkyl substituent is β) is less stable and therefore dehydrates to relieve strain whereas chemical dehydration of



[4] [117] [118]



[119] [120]

this epimer necessitates increased interaction between the C-17 and C-18 substituents prior to formation of the endocyclic double bond. Consequently the epimer in which the smaller (hydroxyl) substituent is closest to the C-18 methyl group is chemically dehydrated fastest, the ease of exocyclic elimination presumably being equal for both epimers.

The dehydration of methandrostenolone[4] with phosphorus oxychloride in pyridine at room temperature was accompanied by much degradation, no A-ring protons being distinguishable in the n.m.r. spectrum of the product. However, this spectrum showed that exocyclic elimination to give the 17-methylene function [117] (τ5.38) was more favourable than endocyclic elimination to the Δ^{16}-double bond [118] (τ4.75). The ratio of exo- to endocyclic elimination was estimated to be ca. 6:1.

A mixture of 17-methyleneandrosta-1,4-dien-3-one and 17-methyleneandrost-4-en-3-one was prepared by the reaction of DDQ with the latter compound and then reacted with phosphorus oxychloride as described above. Once again, degradation occurred and, although the C-4 proton of the Δ^{4}-en-3-one (τ4.27) was clearly visible in the n.m.r. spectrum of the crude product the A-ring protons of the dienone could not be detected. Similarly, the infrared spectrum showed the loss of the cross-conjugated carbonyl function. Thus, although the nature of this degradation is unknown it is specific for the $\Delta^{1,4}$-dien-3-one function and, like the dienone-phenol rearrangement of the A-ring in sulphuric acid, does not take place with a Δ^{4}-en-3-one. In view of the extensive degradation the dehydration of 17-epimethandrosten-

olone was not investigated under these conditions.

3.13.iv. Acetylation

Ananchenko et al.[89] demonstrated a difference between the chemical reactivity of the C-17a epimeric 3-methoxy-17a-methyl-D-homooestra-1,3,5(10),8-tetraen-17a-ols by their reactions with acetic anhydride in pyridine. After reaction at reflux temperature for 2 hours both epimers were partially converted to their acetates. However, after 6 hours the 17aβ-ol was dehydrated to the 17a-methylene compound whereas the 17aα-ol was converted to the acetate.[89]

When methandrostenolone was treated under the same acetylation conditions the n.m.r. spectra of the reaction products after times of 2 hours and 6 hours were the same. The 17β-acetate was the major product with only a trace of dehydration to the 17-methylene compound (ca. 4%). Since methandrostenolone would be expected to dehydrate more readily under these conditions the reaction of 17-epimethandrostenolone was not examined.

Since the product of the acetylation of methandrostenolone under the conditions described above was a non-crystalline, dark-coloured glass alternative methods of acetylation were examined. The lack of reactivity of steroid alcohols containing hindered functions, such as the secondary 11β-hydroxyl group and tertiary hydroxyl groups, to acetylation with acetic anhydride in pyridine at room temperature may be explained by the necessity for a termolecular transition state.[117g] Oliveto et al.[185] found that the use of more drastic conditions, for example heating, for the

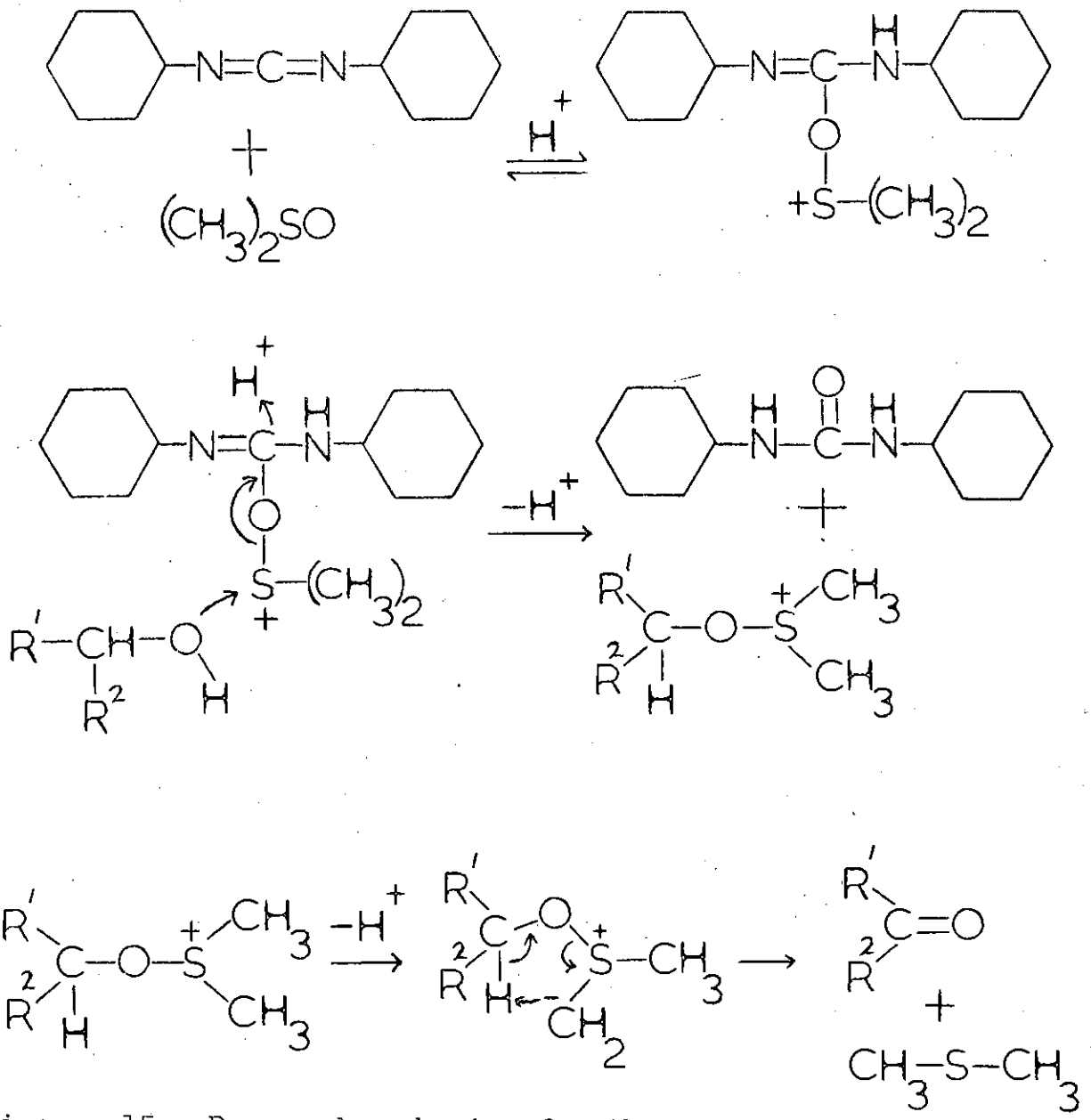


Diagram 15. Proposed mechanism for the oxidation of secondary alcohols by dicyclohexylcarbodiimide and dimethyl sulphoxide.

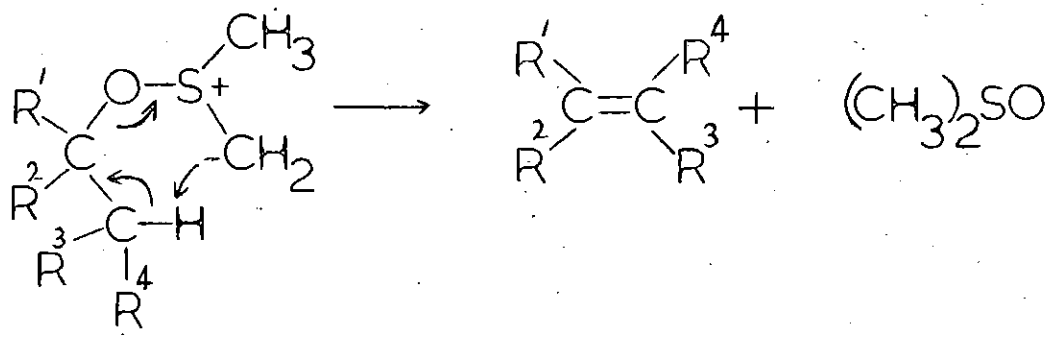


Diagram 16. Proposed mechanism for the dehydration of tertiary steroid alcohols with dicyclohexylcarbodiimide and dimethylsulphoxide.

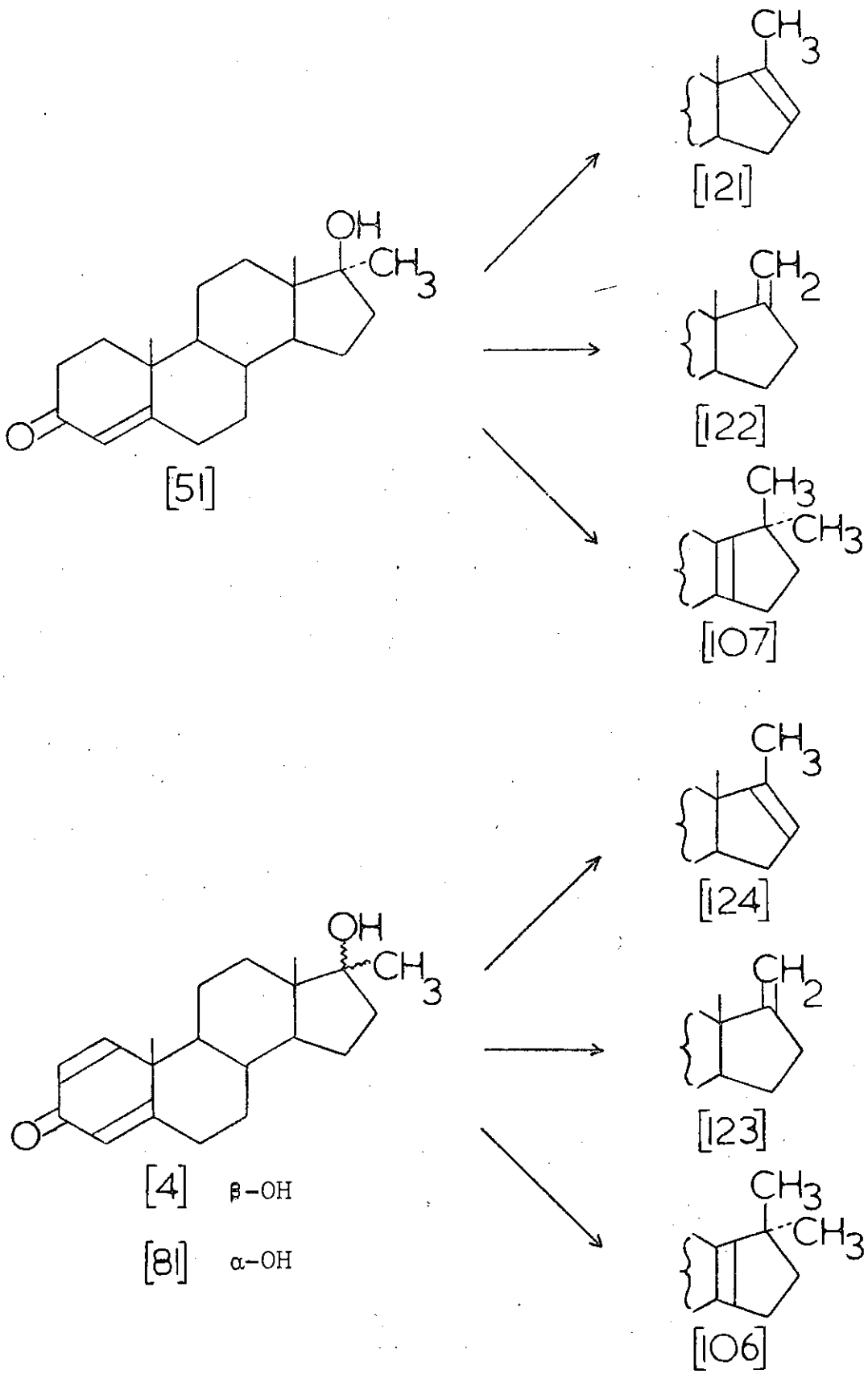
acetylation of the 11β-hydroxyl group caused dehydration. However, the position was readily acetylated[185] at room temperature with an acetylating reagent in the presence of a strong acid catalyst, under which conditions the reactive acetylium species is formed.[117g]

When methandrostenolone was reacted with acetic anhydride in acetic acid with toluene-p-sulphonic acid[185] as catalyst complete reaction occurred at room temperature. Although the n.m.r. spectrum of the product showed that there was no dehydration under these conditions, the τ3.0-3.5 region indicated that a small amount of A-ring rearrangement occurred. The major product was methandrostenolone acetate but, as before, it was not crystalline.

The mass spectrum of this product showed the expected molecular ion of methandrostenolone acetate at m/e 342 and an intense ion at m/e 282, arising from elimination of acetic acid. Ions at m/e 340 and m/e 324 were not rationalisable in terms of this compound. However a low intensity ion at m/e 384 indicated the formation of a small amount of a diacetate, probably 4,17α-dimethyloestra-1,3,5(10)-triene-1, 17-diol diacetate [119]. The ions [120] at m/e 340 and m/e 324 would then arise by elimination of CH_3COH, probably from a cyclic conformation of the acetate, and of acetic acid, respectively, from this diacetate.

3.13.v. Sulphoxide-carbodiimide oxidation

The oxidation of secondary steroid alcohols to ketones has been accomplished with dicyclohexylcarbodiimide and dimethyl sulphoxide[186a] [diagram 15]. The selective oxidation


OH
CH3
O
[51]
CH3
[121]
CH2
[122]
CH3
CH3
[107]
CH3
[124]
CH2
[123]
CH3
CH3
[106]
OH
CH3
O
[4] β-OH
[81] α-OH

of secondary alcohols in the presence of tertiary alcohols has been achieved in some compounds but certain tertiary alcohols have been found to dehydrate very readily.[186b] Moffatt observed[186b] (unpublished experiments) that methyltestosterone[51] was completely dehydrated almost instantaneously to a 3:2 mixture of the Δ^{16}- [121] and 17-methylene [122] compounds. He suggested that this dehydration involved an intramolecular proton abstraction mechanism [diagram 16] similar to that proposed for the oxidation [diagram 15] but with regeneration of dimethyl sulphoxide rather than formation of dimethyl sulphide.[186b]

Prior to examination of the reaction of methandrostenolone under these conditions the reaction of methyltestosterone was carried out. Precipitation of dicyclohexylurea was observed almost immediately after mixing of the reagents but t.l.c. of the product after 2 hours showed that not all the steroid had reacted. Slightly less than 50% of the product was eluted from an alumina column as a low polarity band. The n.m.r. spectrum of this fraction showed that the 17-methylene [122] and Δ^{16}-compounds [121] were present in a 1:1 mixture which accounted for 70% of the total fraction. A third product was identified as 17,17-dimethyl-18-norandrosta-4,13(14)-dien-3-one[107][τ9.05, 9.04 (C-17,17dimethyl), 8.85 (C-19 methyl)]. Unreacted methyltestosterone was then recovered from the column. These results were confirmed by g.l.c., which showed three short retention time peaks in the low polarity fraction.

Since Moffatt[186b] did not include the $\Delta^{13(14)}$-17,17-dimethyl compound [107] among the products of the reaction of methyltestosterone the reaction

was repeated without dicyclohexylcarbodiimide and without dimethyl sulphoxide and dicyclohexylcarbodiimide. T.l.c. of the products of both reactions showed no spots other than that corresponding to methyltestosterone. Consequently, 17,17-dimethyl-18-norandrosta-4,13(14)-dien-3-one must be a product of the dimethyl sulphoxide-dicyclohexylcarbodiimide reaction.

When methandrostenolone[4] was reacted with dimethyl sulphoxide and dicyclohexylcarbodiimide for one hour less than 10% of the sample was converted to a mixture of the 17-methylene [123] and Δ^{16}-compounds [124]. No $\Delta^{13(14)}$-17,17-dimethyl compound [106] was detected. The reaction was repeated and t.l.c. after three hours showed that very little conversion had occurred but after sixty hours all the steroid had reacted. The product now contained a 1:1 mixture of the 17-methylene [123] and Δ^{16}-compounds [124] and 20-30% 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one[106]. Under similar conditions 17-epimethandrostenolone[81] reacted completely within three hours to give a mixture containing the 17-methylene [123], Δ^{16}- [124] and $\Delta^{13(14)}$-17,17-dimethyl [106] compounds in the ratio ca. 5:3:2.

The reactions of methandrostenolone without dicyclohexylcarbodiimide and without dimethyl sulphoxide and dicyclohexylcarbodiimide were examined. T.l.c. of both products showed a very intense spot corresponding to methandrostenolone and a very faint low polarity spot which could not have accounted for the 20-30% formation of 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one in the reaction with dicyclohexylcarbodiimide.

3.13.vi. Phosphorus pentachloride

The products of the reactions of secondary 3β-, 7β- and 17β- alcohols with phosphorus pentachloride are the epimeric α-chloro compounds.[117h] The reaction involves the formation of a colinear O.....C.....Cl transition state and consequently alcohols, such as 2α- and 6α-hydroxy steroids react by an S_Ni mechanism with retention of configuration because of the unfavourable interactions which would be involved in the formation of the S_N2 transition state.[117h]

The tertiary alcohol, 12α-hydroxy-12-methyltigogenin acetate has been treated with phosphorus pentachloride to give the 12β-chloro-12-methyl derivative.[187] Although the reaction appears to proceed by an S_N2 mechanism it has been reported that deliberate attempts to produce the C-12 carbonium ion resulted in degradation of the steroid.[117i]

When methandrostenolone was reacted with phosphorus pentachloride in ether[187] at room temperature for 48 hours extensive degradation occurred, indicated by the poorly resolved n.m.r. spectrum and loss of the carbonyl absorption in the infrared spectrum of the product. A similar reaction of methandrostenolone in chloroform[188] containing calcium carbonate for 1 hour at 0^o gave predominantly unreacted steroid. The less polar 17,17-dimethyl-18-norandrosta-1, 4,13(14)-trien-3-one was also isolated but the mass spectra of both products showed no trace of a compound in which the 17β-hydroxyl group had been substituted by a chlorine atom.

The failure of methandrostenolone to form a 17α-chloro-17-methyl product by reaction with phosphorus pentachloride was probably due to steric hindrance which would prevent the

formation of a colinear O.....C.....Cl transition state.

3.13.vii. Conclusion

The results described in this section showed that any reaction of methandrostenolone, such as that in sulphuric acid, in which a C-17 carbonium ion was formed led to the 17,17-dimethyl-18-nor-$\Delta^{13(14)}$- derivative by dehydration with C-18 methyl migration. Epimerisation could only proceed from the C-17 carbonium ion if this migration could be prevented. A reaction such as that of methandrostenolone with phosphorus pentachloride, in which a C-17 epimeric chloro compound might have been produced by a mechanism not involving a carbonium ion presumably did not proceed because of steric hindrance to the formation of the required transition state. Consequently the epimerisation of methandrostenolone by chemical means was impossible whereas it is feasible that an enzyme, by virtue of its stereochemistry, might block C-18 methyl migration and introduce a hydroxyl group from the α-side of the molecule thus forming 17-epimethandrostenolone.

3.14. Experimental section

3.14.i. Reaction of methandrostenolone with sodium methoxide

Sodium (ca. 200mg) was added to a solution of methandrostenolone (102.5mg) in methanol (50ml) and refluxed for 2 hours under nitrogen, during which time the colour of the solution darkened. Methanol was removed by evaporation in vacuo and the residue was dissolved in ether. The solution was washed with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a brown glass (127.3mg); t.l.c. showed a spot with the same polarity and colour reaction as methandrostenolone and faint traces of two more polar components; n.m.r. (100 M Hz) τ9.07 (C-18 methyl), 8.82 (C-20 methyl), 8.75 (C-19 methyl), 3.94 (d, 2 Hz, 4-H), 3.79 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.94 (d, 10 Hz, 1-H) identified the product as unchanged methandrostenolone.

3.14.ii. Reaction of methandrostenolone with sodium hydroxide in ethanol[189]

A solution of methandrostenolone (105.7) in ethanol (30ml) containing 20% sodium hydroxide solution (2 drops) was refluxed for 2 hours. The ethanol was removed by evaporation in vacuo and the residue dissolved in ether, washed with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a brown glass (126.6mg). T.l.c. and n.m.r. spectroscopy (100 M Hz) indicated that the product was methandrostenolone; τ9.07 (C-18 methyl) 8.82 (C-20 methyl), 8.76 (C-19 methyl), 3.93

(d, 2 Hz, 4-H), 3.78 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.94 (d, 10 Hz, 1-H).

3.14.iii. Reaction of methandrostenolone with aqueous sulphuric acid

Methandrostenolone (46.8mg) and 20% aqueous sulphuric acid (10ml) were heated at 100° for 2 hours. When cool the aqueous phase was extracted twice with ether, the combined ether extracts were washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a brown glass (53.5mg), the major component of which was 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one; ν_{max} 1665cm^{-1}, n.m.r. (100 M Hz) τ9.08, 9.04 (C-17,17 dimethyl), 8.80 (C-19 methyl), 3.87 (d, 2 Hz, 4-H), 3.72 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.84 (d, 10 Hz, 1-H); g.l.c., Retention time, 5.64 minutes (235°). 3-Hydroxy-1,17,17-trimethyl-18-noroestra-1,3,5(10),13(14)-tetraene and 1-hydroxy-4,17,17-trimethyl-18-noroestra-1,3,5(10),13(14)-tetraene were also formed; n.m.r. τ3.5-3.0 (aromatic protons); g.l.c., Retention times, 13.36 and 14.60 minutes (235°).

The crude product was chromatographed on alumina (10g) to give 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one as a low polarity glass; ν_{max} 1665cm^{-1}; mass spectrum, m/e 282 (100%), m/e 267 (71%), m/e 239 (3%) [see figure 11b].

3.14.iv. Reaction of 17-epimethandrostenolone with aqueous sulphuric acid

A suspension of 17-epimethandrostenolone (8.9mg) in 20% aqueous sulphuric acid (2ml) was heated at 100° for

2 hours. The product, a brown glass (12.2mg) was extracted as described in section 3.14.iii. T.l.c. showed that complete reaction had occurred to give a low polarity product the n.m.r. spectrum of which was identical to that of the product of the corresponding reaction of methandrostenolone (section 3.14.iii).

3.14.v. Reaction of methyltestosterone with aqueous sulphuric acid

Methyltestosterone (53.1mg) and 20% aqueous sulphuric acid (10ml) were heated at 100° for 1½ hours and the product (52.1mg), a brown glass, was extracted as described in section 3.14.iii. The major product (ca. 86%) was 17,17-dimethyl-18-norandrosta-4,13(14)-dien-3-one; t.l.c., low polarity; n.m.r. (100 M Hz) τ9.06, 9.04 (C-17,17 dimethyl), 8.85 (C-19 methyl), 4.24 (4-H) with unreacted methyltestosterone (ca. 14%); n.m.r. τ9.09 (C-18 methyl), 8.79 (C-19 methyl), 8.79 (C-20 methyl), 4.25 (4-H).

3.14.vi. Reaction of methandrostenolone with sulphuric acid in acetic acid

20% Aqueous sulphuric acid (10ml) was added to a solution of methandrostenolone (106.8mg) in glacial acetic acid (2ml). The light precipitation was dissolved by the addition of glacial acetic acid (6ml) and the solution was heated at 100° whereupon a red precipitate formed and was not redissolved by the addition of further glacial acetic acid (5ml). After 2 hours the mixture was allowed to cool, extracted with ether and the ether solution neutralised with saturated sodium bicarbonate solution, washed with water,

dried over anhydrous magensium sulphate and evaporated to dryness. The product, a dark brown glass (110.6mg) contained no unreacted methandrostenolone. The major component was 1-hydroxy-4,17,17-trimethyl-18-noroestra-1,3,5(10),13(14)-tetraene; n.m.r. (100 M Hz) τ9.02, 8.99 (C-17,17 dimethyl), 7.86 (C-4 methyl), 5.04 (broad, 1-OH), 3.48, 3.14 (d,d, 8 Hz, 8 Hz, 2-H, 3-H) with some 3-hydroxy-1,17,17-trimethyl-18-noroestra-1,3,5(10),13(14)-tetraene; n.m.r., τ3.5-3.0 (small peaks).

3.14.vii. Reaction of methandrostenolone with phosphorus oxychloride[89]

Freshly distilled phosphorus oxychloride (0.05ml) was added to a solution of methandrostenolone (112.5mg) in dry pyridine (2ml), whereupon fuming occurred and a yellow colour was generated. After 18 hours at room temperature the dark red solution was poured into a mixture of ice and water and the steroid extracted into chloroform, washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a dark brown glass (80.7mg), which contained no carbonyl band in its infrared spectrum; n.m.r. (100 M Hz) τ5.38 (17 = CH_2), 4.75 (16-H), 6:1; further purification and characterisation was not undertaken because of the tar-like nature of the product, which had obviously suffered extensive degradation.

3.14.viii. Synthesis of 17-methyleneandrosta-1,4-dien-3-one

A solution of 17-methyleneandrost-4-en-3-one (49.4mg) and DDQ (42.6mg) in dioxane (12ml) was refluxed for 20 hours.

The dioxane was removed by evaporation in vacuo and the residue was dissolved in benzene, filtered through alumina (1g) and evaporated to dryness to yield the crude product (54.3mg) which was chromatographed on alumina (2g). Elution with benzene gave a mixture (31.7mg) containing equal amounts of the starting material and 17-methyleneandrosta-1,4-dien-3-one; ν_{max} 1665cm^{-1}; n.m.r. (60 M Hz) τ9.18 (C-18 methyl), 8.77 (C-19 methyl) $\Delta^{1,4}$-dien-3-one and τ9.16 (C-18 methyl), 8.80 (C-19 methyl) Δ^{4}-en-3-one.

3.14.ix. Reaction of 17-methyleneandrosta-1,4-dien-3-one with phosphorus oxychloride[89]

Phosphorus oxychloride (0.025ml) was added to a solution of the product of the previous reaction (30mg) in dry pyridine (1ml). After 18 hours at room temperature the reaction mixture was poured into a mixture of ice and water and the steroids were extracted into chloroform, washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a brown glass (27mg); ν_{max} 1675cm^{-1}, no band at 1665cm^{-1}; n.m.r. (100 M Hz) τ 5.36 (17 = C$\underline{H}_2$), 4.27 (4-$\underline{H}$), no protons in τ2-3 region ($\Delta^{1,4}$-dien-3-one).

3.14.x. Acetylation of methandrostenolone

a. Acetic anhydride and pyridine at reflux temperature for 2 hours[89]

A solution of methandrostenolone (49.6mg) in pyridine (0.6ml) and acetic anhydride (0.6ml) was refluxed for 2 hours, cooled and poured into dilute acetic acid (1:4, 20ml). After 30 minutes the steroid was extracted into chloroform, washed with saturated sodium bicarbonate solution and with water,

dried over anhydrous magnesium sulphate and evaporated to dryness to give a brown glass (55.6mg); ν_{max} 1730,1665cm^{-1}; n.m.r. (100 M Hz) τ9.09 (C-18 methyl), 8.76 (C-19 methyl), 8.62 (C-20 methyl), 8.03 (CO.C$\underline{H}_3$), 3.94 (d, 2 Hz, 4-H), 3.79 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.95 (d, 10 Hz, 1-H). A signal at τ5.37 showed that the product contained a trace of the 17-methylene compound arising from exocyclic dehydration (ca. 4%).

b. Acetic anhydride and pyridine at reflux temperature for 6 hours[89]

Methandrostenolone (53.5mg) was reacted by the procedure described above, except that the reaction time was increased to 6 hours. The t.l.c., infrared and n.m.r. spectra of the product (57.1mg), a brown glass, were similar to those of the product of the 2 hour reaction. The amount of the 17-methylene compound (ca. 4%) in the product had not increased after the longer reaction time.

c. Acetic anhydride and toluene-p-sulphonic acid at room temperature[185]

Acetic anhydride (0.08ml) and toluene-p-sulphonic acid (4mg) were added to a solution of methandrostenolone (44.5mg) in acetic acid (0.4ml). After 7 hours t.l.c. indicated that all the methandrostenolone had reacted. Water was added and the steroid extracted into ether, neutralised with saturated sodium bicarbonate solution, washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a brown glass (58.8mg). The infrared and n.m.r. spectra of the product were similar to

those of the preceding acetylation reactions except that no dehydration had occurred and there was a small amount of A-ring rearrangement (τ3.5-3.0, small peaks). The major product, methandrostenolone acetate was of low polarity compared to methandrostenolone and there was a trace of a less polar component. Mass spectrum, m/e 342 (M^+, 12%), m/e 282 (M-AcOH, 83%) methandrostenolone acetate; m/e 384 (3%), m/e 340 (15%), m/e 324 (17%) probably 4,17α-dimethyl-oestra-1,3,5(10)-triene-1,17-diol diacetate.

3.14.xi. Reaction of methyltestosterone with dimethyl sulphoxide and dicyclohexylcarbodiimide[186]

Methyltestosterone (502mg) was dissolved in a mixture of dimethyl sulphoxide (6ml) and benzene (20ml) containing dicyclohexylcarbodiimide (1.02g). Pyridine-trifluoroacetic acid adduct (50mg)was added and the reaction vessel stoppered. After 2 hours ether and oxalic acid (0.541g) were added. When gas evolution had ceased water was added and the precipitated dicyclohexylurea removed by filtration. The filtrate was washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield the product (0.579g) which contained unreacted methyltestosterone and a less polar band. The crude product was chromatographed on 5% deactivated alumina (17g) and a white crystalline material (234mg) was eluted with petrol-benzene (3:1 to 3:2). The n.m.r. spectrum (100 M Hz) showed that the fraction contained a 1:1 mixture of 17-methylene (τ5.36) and Δ^{16}-compounds (τ4.74) which accounted for 70% of the sample. The methyl signals were assigned to 17,17-dimethyl-18-

norandrosta-4,13(14)-dien-3-one, τ9.05, 9.04 (C-17,17 dimethyl), 8.85 (C-19 methyl); 17-methyleneandrost-4-en-3-one, τ9.18 (C-18 methyl), 8.79 (C-19 methyl) and 17-methylandrosta-4, 16-dien-3-one, τ9.22 (C-18 methyl), 8.79 (C-19 methyl), 8.36 (C-20 methyl). Unreacted methyltestosterone was eluted from the column with benzene.

3.14.xii. Reaction of methandrostenolone with dimethylsulphoxide and dicyclohexylcarbodiimide[186]

Methandrostenolone (53.0mg) was dissolved in a mixture of benzene and dimethyl sulphoxide (3:1, 1ml) containing dicyclohexylcarbodiimide (0.1g). Pyridine-trifluoroacetic acid complex (5mg) was added and the reaction vessel was stoppered. After 1 hour at room temperature the reaction mixture was diluted with ether and oxalic acid (65.7mg) was added. The product (63.6mg) was isolated as described in the previous section and t.l.c. indicated that the major component was unreacted methandrostenolone. No other compounds were detectable in the n.m.r. spectrum of the crude product. Chromatography on 5% deactivated alumina (4g) and elution with petrol-benzene (3:2) gave a low polarity fraction (4mg); n.m.r. (100 M Hz) τ5.37 (17 = $C\underline{H}_2$), 4.75 (16-$\underline{H}$) 1:1 mixture. The methyl signals could not be assigned because of the low intensities due to the small sample size.

The reaction was repeated for 60 hours. T.l.c. of the reaction mixture after 3 hours showed that very little reaction had occurred whereas after 60 hours most of the methandrostenolone had reacted to give a low polarity band.

The product, a yellow glass, contained a 1:1 mixture of the 17-methylene (τ5.36) and Δ^{16}-compounds (τ4.74), accounting for ca. 70% of the sample. The methyl signals in the n.m.r. (100 M Hz) spectrum of the product were assigned to 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one, τ9.07, 9.03 (C-17,17 dimethyl), 8.78 (C-19 methyl), 17-methyleneandrosta-1,4-dien-3-one, τ9.14 (C-18 methyl), 8.75 (C-19 methyl) and 17-methylandrosta-1,4,16-trien-3-one, τ9.19 (C-18 methyl), 8.75 (C-19 methyl), 8.36 (C-20 methyl). G.l.c. indicated that the ratio of the 17,17-dimethyl-(5.56 minutes),17-methylene-(7.04 minutes) and 17-methyl-Δ^{16}-(6.52 minutes) products was ca. 0.4:1:1 (235°C).

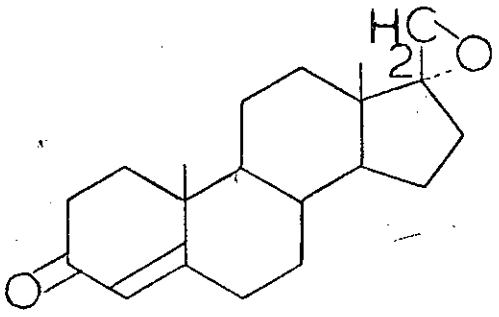
3.14.xiii. Reaction of 17-epimethandrostenolone with dimethyl sulphoxide and dicyclohexylcarbodiimide[186]

17-Epimethandrostenolone (6.5mg) was dissolved in a mixture of benzene and dimethyl sulphoxide (3:1, 0.2ml) containing dicyclohexylcarbodiimide (20mg). Pyridine-trifluoroacetic acid adduct (1mg) was added and the reaction vessel stoppered. After 3 hours, t.l.c. of the reaction mixture indicated that all the steroid had reacted. Ether and oxalic acid (11.8mg) were added and the product, a yellow glass (17.1mg) isolated as described in section 3.14.xi. The n.m.r. spectrum of the product was similar to that of the preceding reaction of methandrostenolone except that the ratio of 17-methylene- to Δ^{16}- products was greater than unity. The ratio of 17,17-dimethyl-(5.72 minutes), 17-methylene-(7.12 minutes) and 17-methyl-Δ^{16}-(6.60 minutes) compounds was ca. 2:5:3 from g.l.c. at 235°C.

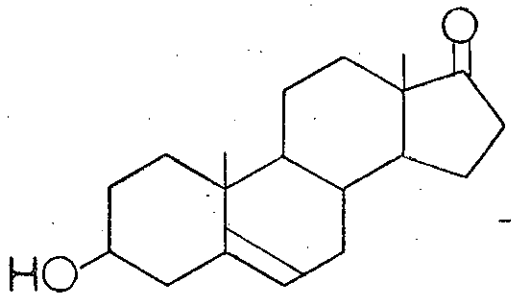
3.14.xiv. Reaction of methandrostenolone with phosphorus pentachloride

a.[188] Phosphorus pentachloride (ca. 100-200mg), which had been sublimed at 80-90° (ca. 0.04 mm Hg) was added to a solution of methandrostenolone (104.9mg) in chloroform (20ml) containing calcium carbonate (521.5mg) in a dry box. The solid reagents had been dried over phosphorus pentoxide in vacuo and the chloroform over molecular sieve. The flask was stoppered and removed from the dry atmosphere into an ice bath. Upon addition of the phosphorus pentachloride the chloroform solution became orange-coloured. After 1 hour at 0° saturated sodium bicarbonate solution was added to the reaction mixture, which was then extracted with chloroform. The organic phase was washed repeatedly with base to neutralise the acid produced by slow hydrolysis of the excess reagent. It was then washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a dark rust-coloured glass (145.6mg). T.l.c. showed that the product contained two components, the major of the same polarity as methandrostenolone and the other of lower polarity. Preparative t.l.c. of the crude product gave two fractions; the least polar was identified as 17,17-dimethyl-18-norandrosta-1,4,13(14)-trien-3-one (16.1mg); n.m.r. (100 M Hz) τ9.07, 9.03 (C-17,17 dimethyl), 8.78 (C-19 methyl), 3.90 (d, 2 Hz, 4-H), 3.75 (d of d, J_{12} 10 Hz, J_{24} 2 Hz, 2-H), 2.85 (d, 10 Hz, 1-H); mass spectrum, m/e 282 (65%), m/e 267 (29%), m/e 239 (6%) and the other as unreacted methandrostenolone (40.6mg), from its n.m.r. and mass spectra.

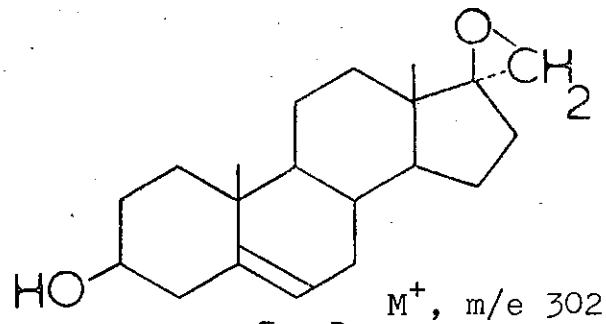
b.[187] Freshly sublimed phosphorus pentachloride (ca. 100-200mg) was added to a solution of dry methandrostenolone (66.3mg) in sodium-dried ether (20ml) in a dry box. A yellow colour was generated when the reagent was added but after 1½ hours at 0° it had not dissolved. The reaction was thus left at room temperature for 48 hours when a dark-coloured solution was obtained. Ether and water were added, the organic layer was washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a dark rust-coloured glass (94.7mg). T.l.c. indicated that the crude product contained three components of low polarity compared to methandrostenolone. The carbonyl function, ν_{max}1665cm^{-1} was absent from the infrared spectrum and the n.m.r. spectrum showed that much degradation had occurred; consequently the products were not further examined.



[68]

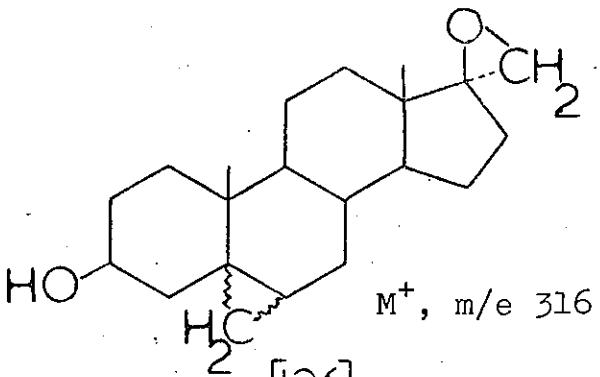


[48]



M^+, m/e 302

[125]



M^+, m/e 316

[126]

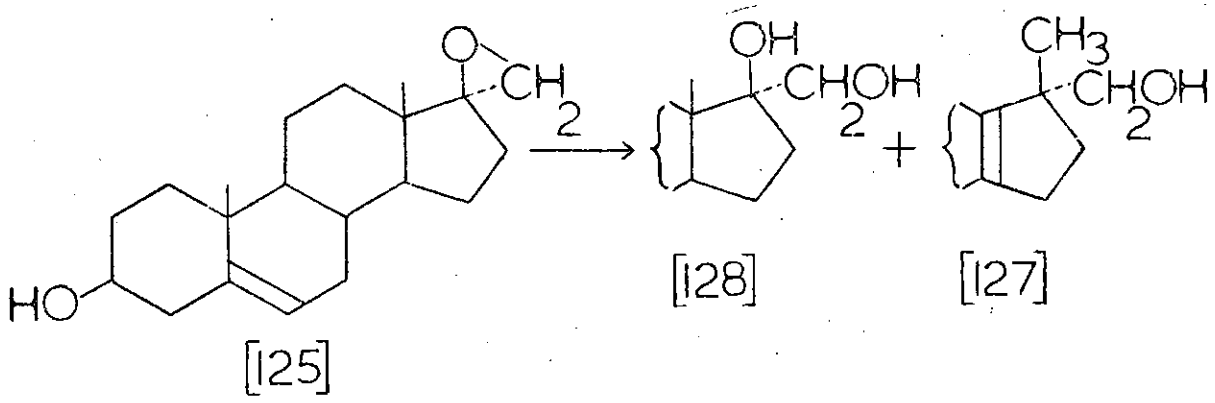
3.15. Miscellaneous reactions

3.15.i. The reactions of 17α,20- and 17β,20-epoxides with boron trifluoride

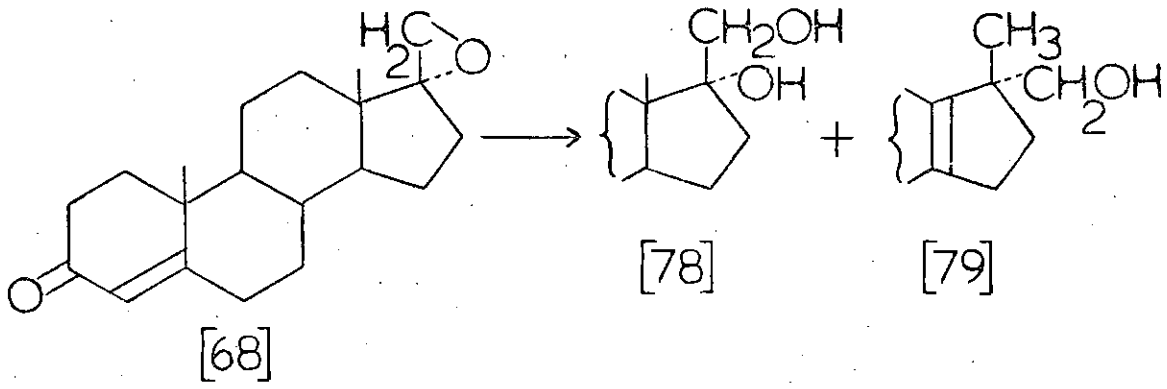
17α,20-Oxido-21-norpregn-4-en-3-one[68] was synthesised during the preparation of 17-epimethandrostenolone (section 3.1). In order to compare the reactions of 17α,20- and 17β,20-epoxides with boron trifluoride-etherate 17β,20-oxido-21-norpregn-5-en-3β-ol[125] was prepared from dehydroepiandrosterone[48] by the method of Franzen and Driessen.[190] The product of the epoxidation reaction appeared to be homogeneous by t.l.c. and its n.m.r. spectrum contained the expected two proton pair of doublets at τ7.40 and 7.10 (J = 6 Hz). However the mass spectrum at 120° contained not only the molecular ion of the epoxide (m/e 302, 100%) but also an intense ion at m/e 316 (75%). At higher temperatures, 140, 160 and 170° the intensity of the latter ion fell to 20, 7 and 3% of the m/e 302 ion, respectively.

Dimethylsulphonium methylide does not usually react to generate cyclopropanes[112e] whereas dimethyloxosulphonium methylide has been shown to react with α,β-unsaturated ketones to give cyclopropyl ketones.[112f] Since the component with M^+, m/e 316 could only be detected by mass spectrometry at 120° a small proportion of the Δ^5-en-3β-ol may however react with dimethylsulphonium methylide to form a 5,6-cyclopropyl-17β,20-epoxide[126](M^+, m/e 316).

The preparation of 17β,20-oxido-21-norpregn-4-en-3-one by the Oppenauer oxidation of the Δ^5-en-3β-ol has been reported.[191] This reaction was attempted but the n.m.r.



[125] [128] [127]



[68] [78] [79]

spectrum of the product showed that degradation of the epoxide function had occurred.

17β,20-Oxido-21-norpregn-5-en-3β-ol[125] was reacted with boron trifluoride-etherate in acetamide at ca 100° for 1¾ hours. The n.m.r. spectrum of the crude product contained two pairs of doublets at τ6.70, 6.60 (J_{AB} 10 Hz) and at τ5.83, 5.73 (J_{AB} 11 Hz), the former pair of which were assigned to the C-20 methylene protons of 17α-hydroxymethyl-17-methyl-18-norandrosta-5,13(14)-dien-3β-ol[127] (cf. 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one, described in section 3.16.ii., τ6.70, 6.60, J_{AB} 10 Hz, $-C\underline{H}_2OH$). Since the 17α,20-epoxide function[68] was shown to open under acid conditions to give a 17α-hydroxy-17-hydroxymethyl compound [78] (section 3.1.v.), τ6.43, 6.38, J_{AB} 11 Hz, $-C\underline{H}_2OH$ the pair of doublets at τ5.83, 5.73 were ascribed to the C-20 methylene protons of 17α-hydroxymethylandrost-5-ene-3β,17-diol[192][128]. This compound [128] and the 17α-hydroxymethyl-17-methyl diol [127] were present in the crude product in approximately equal proportions as calculated from the n.m.r. spectrum. However crystallisation from acetone gave pure 17α-hydroxymethyl-17-methyl-18-norandrosta-5,13(14)-dien-3β-ol[127], τ6.70, 6.60, $-C\underline{H}_2OH$. Reaction of the 17β,20-epoxide [125] with boron trifluoride in benzene for 24 hours gave a red glass, the n.m.r. spectrum of which showed that considerable degradation had occurred although 17α-hydroxymethyl-17-methyl-18-norandrosta-5,13(14)-dien-3β-ol[127], τ6.65 (small peak, $-C\underline{H}_2OH$), 9.02 (C-17 methyl), 9.02 (C-19 methyl) was probably present. Within five minutes of the

addition of boron trifluoride the reaction solution had changed from colourless through deep orange and deep red to violet, accompanied by the deposition of black material. Consequently the products were not further investigated.

Reaction of 17α,20-oxido-21-norpregn-4-en-3-one[68] with boron trifluorode-etherate in acetamide under the conditions described above gave only 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one[79]. The n.m.r. spectrum of the crude product showed no trace of 17α-hydroxy-17-hydroxymethylandrost-4-en-3-one[78]. When the reaction was carried out in benzene for 24 hours the n.m.r. spectrum of the crude product showed that degradation had occurred although the 17α-hydroxymethyl-17-methyl compound [79] was formed. The reaction mixture quickly changed from pale yellow to orange, rust red and black with deposition of material. The reaction was repeated in benzene for two minutes, when a yellow solution was obtained. The n.m.r. spectrum of the crude product showed only 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one[79], although t.l.c. indicated traces of several other components.

Boron trifluoride is a Lewis acid and is thus able to react with a polar solvent to form a Lewis base accompanied by the generation of protons. Protonation of an epoxide leads to an electron deficient carbon centre which is readily attacked by an anion or solvent molecule with cleavage of the epoxide ring. The product of such a reaction is generally a trans diaxially substituted compound with the epoxide oxygen forming an axial hydroxyl group corresponding

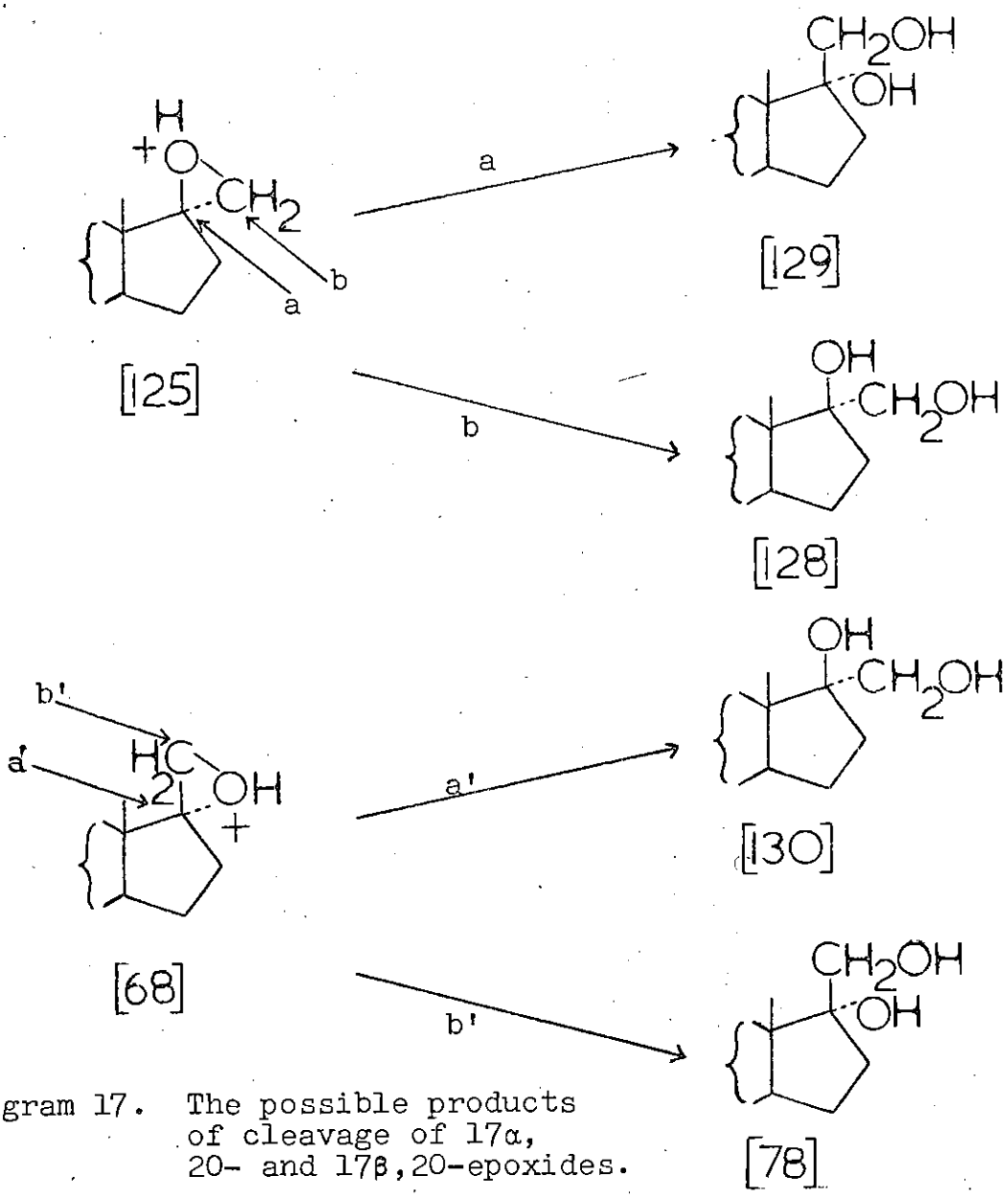
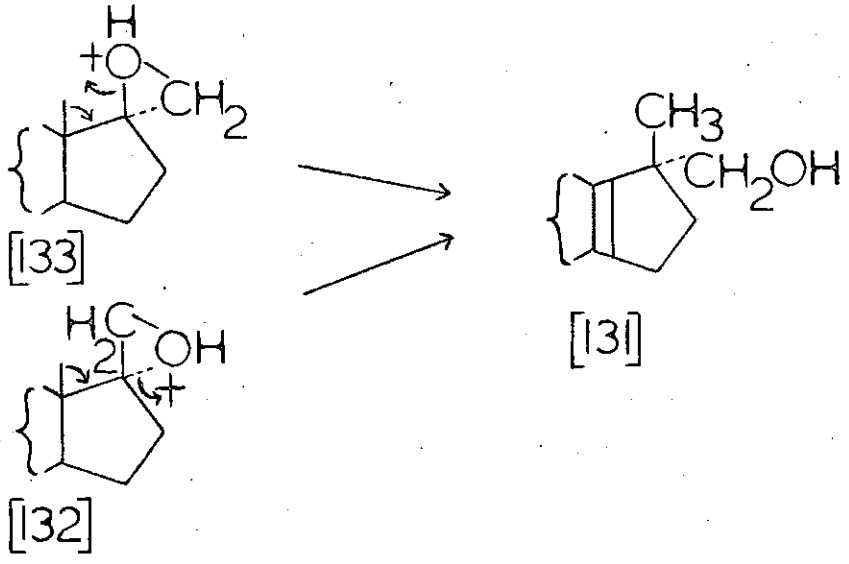


Diagram 17. The possible products of cleavage of 17α, 20- and 17β,20-epoxides.



to the configuration of the epoxide. Attack by a nucleophile, in this case OH^-, at the 17-position [a. in diagram 17] of the 17β,20-epoxide [125] would give the 17α-hydroxy-17-hydroxymethyl product [129] whereas attack at C-20 [b. in diagram 17] would lead to the epimeric product [128] in which the epoxide oxygen is pseudoequatorial. This latter is, indeed, the observed product, showing that the lack of steric hindrance to attack at C-20 compared to C-17, and the only pseudoequatorial nature of the epoxide oxygen in the product outweighs the requirement for diaxial ring cleavage.

In the case of the 17α,20-epoxide [68] attack at C-17 [a', in diagram 17], presumably hindered by the C-18 methyl group would give a 17β-hydroxy-17-hydroxymethyl product [130] whereas attack at C-20 [b'. in diagram 17] would give the epimeric configuration [78] in which the epoxide oxygen forms a pseudoaxial hydroxyl group. This is the product formed by acid-catalysed cleavage of the 17α,20-epoxide (section 3.1.v), although in the boron trifluoride-catalysed reaction only 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one[79] was formed.

The formation of products with the 17α-hydroxymethyl-17-methyl-18-nor-$\Delta^{13(14)}$-ene systems [131] from both 17α,20-[132] and 17β,20-epoxides[133] in acetamide and particularly in benzene is not surprising since the tendency for migration of the C-18 methyl group to C-17 under conditions which tend to produce a positive centre at the 17-position has been discussed already (section 3.13).

3.15.ii. The reaction of 17α-hydroxy-17-hydroxymethyl-androst-4-en-3-one with sulphuric acid

When a mixture of 17α-hydroxy-17-hydroxymethylandrost-4-en-3-one[78] and aqueous sulphuric acid was heated at 100° a red-green fluorescence was observed. N.m.r. spectroscopy showed that the major product after 2 hours was 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one [79], τ6.70, 6.60, $-C\underline{H}_2OH$, accompanied by the unreacted 17α-hydroxy-17-hydroxymethyl compound. However t.l.c. of the crude product showed a complicated mixture of spots similar to that observed after the attempted epoxidation of 17-methyleneandrost-4-en-3-one under excessively acid conditions, described in section 3.1.v. Consequently the fluorescence observed in the latter reaction was probably also due to the generation of 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one.

3.15.iii. The attempted preparation of 17α-phenyl-5α-androstane-3β,17-diol

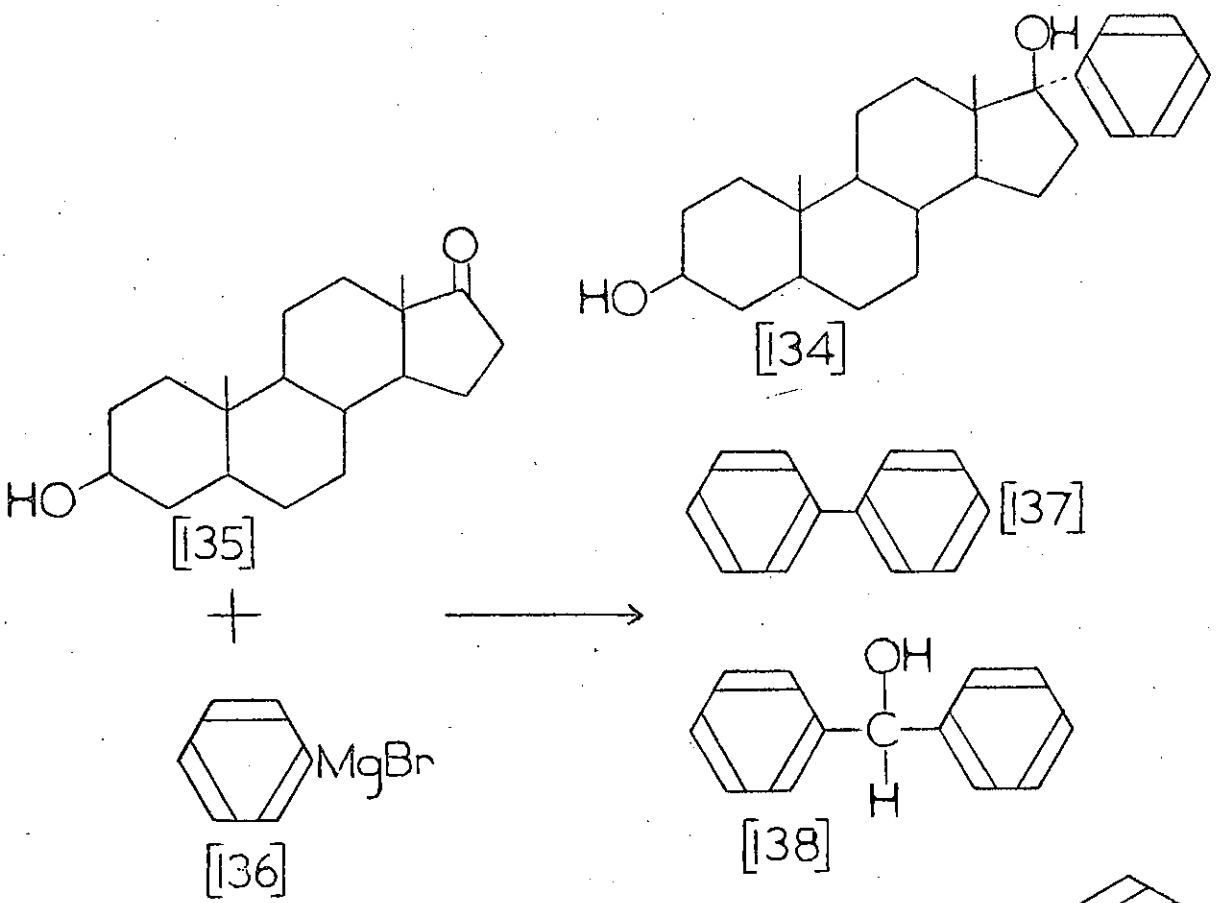
The synthesis of 17α-phenyl-5α-androstane-3β,17-diol [134] was attempted so that the reactions of a tertiary 17β-hydroxy-17-phenyl centre might be compared with those of the 17β-hydroxy-17-methyl function of methandrostenolone (described in section 3.13).

T.l.c. and n.m.r. spectroscopy of the crude product isolated after the reaction of a refluxing mixture of epi-androsterone [135] and phenylmagnesium bromide [136] in ether for 15 minutes indicated that little, if any, reaction had occurred. Consequently, the reaction was repeated with the

use of tetrahydrofuran as the solvent for the steroid and the reaction time was extended to 17½ hours. When the steroid solution was added dropwise to the brown ethereal solution of the Grignard reagent precipitation occurred, which dissolved on swirling. Considerable heat was evolved during mixing of the reagents. When all the steroid had been added a precipitate was formed which did not dissolve when the mixture was heated. Approximately half of the ether used for preparation of the phenylmagnesium bromide was removed by distillation and the mixture was then refluxed for 17½ hours.

The weight of the crude product, a viscous orange oil (3.05g) obtained after destruction of magnesium complexes with ice-cold sulphuric acid was ca. 150% in excess of the theoretical yield (1.29g), assuming 100% formation of the desired product. Crystallisation gradually occurred but crystals could not be isolated by filtration and so the crude product was chromatographed on alumina. A mixture of two low polarity compounds was eluted with petrol and with benzene-petrol (1:1). T.l.c. of the mixture showed two spots, both of which were visible under ultraviolet light but only one, the minor, more polar component was visible, as a yellow spot, after sulphuric acid development. The n.m.r., infrared and mass spectra of this fraction enabled identification of the major component as diphenyl [137].

The more polar component of the previous fraction was obtained by further elution with benzene-petrol (1:1). The infrared spectrum of this compound contained hydroxyl and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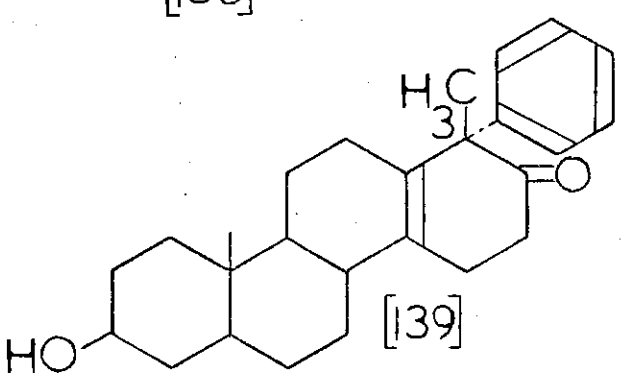
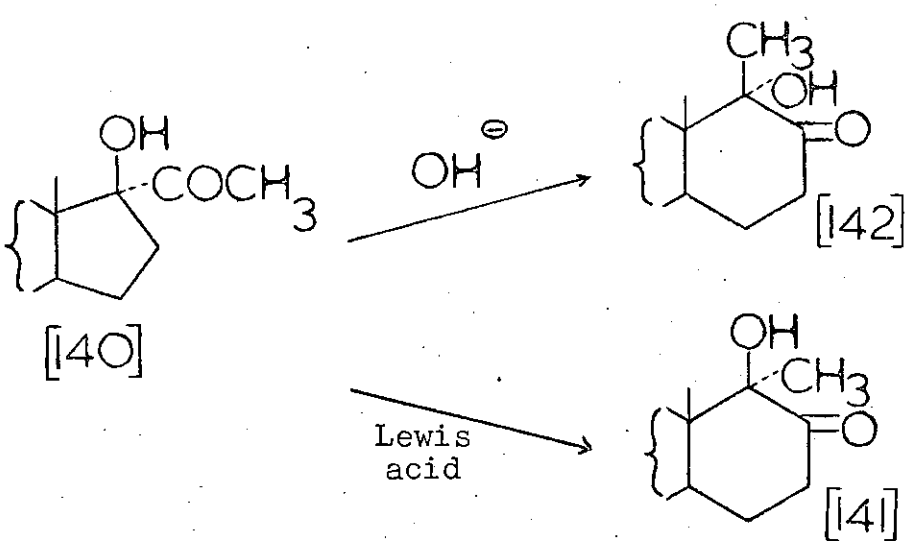
[134]
O
HO
[135]
[137]
OH
C
H
[138]
MgBr
[136]


H3C
O
HO
[139]


OH
COCH3
[140]
OH⊖
CH3
OH
O
[142]
Lewis
acid
OH
CH3
O
[141]

aromatic absorptions and the n.m.r. spectrum showed that three types of protons were present in the ratio 1:1:10, the latter being aromatic protons. Mass spectrometry indicated that the molecular weight of the compound was 184 and the only structure consistent with all the information was that of diphenylcarbinol [134](lit.,[193] m.p. 69°). The melting point of the fraction, 62.5-63.5°, was probably low because of contamination from diphenyl.

Elution with benzene and then with ethanol gave a fraction containing several components, the major of which was obtained pure by crystallisation from acetone. This compound was visible under ultraviolet light and gave a bright orange spot (R_f 0.46) which was slightly less polar than epiandrosterone (R_f 0.41, purple spot). Its infrared spectrum indicated that it contained a hydroxyl group, ν_{max} 3350cm^{-1} together with a carbonyl function in a six-membered ring, ν_{max}1720cm^{-1}. The n.m.r. spectrum showed that the C-3 hydroxyl group was present, τ6.40, 3α-H, and the presence of a series of small doublets between τ7.75 and 7.00 suggested that the carbonyl group was in the D-ring (cf. similar absorptions in the n.m.r. spectra of epiandrosterone and androsterone). The n.m.r. spectrum also showed that the compound contained five aromatic protons. Mass spectrometry gave a molecular weight of 378, ten in excess of that of 17α-phenyl-5α-androstane-3β,17-diol. The only structure which appeared to be consistent with all the data was that of D-homo-3β-hydroxy-17aβ-methyl-17a-phenyl-18-norandrost-13(14)-en-17-one[139]. Also consistent with this structure were the formation of an acetate of molecular weight, 420,

and its complete lack of reaction with aqueous sulphuric acid, with a mixture of sulphuric and acetic acids and with dimethyl sulphoxide-dicyclohexylcarbodiimide under the conditions described in section 3.13.

A very small fraction was finally removed from the column with ethanol. This fraction contained several components, only one of which was visible under ultraviolet light and the n.m.r. spectrum indicated the presence of aromatic protons and the 3β-hydroxyl group. The mass spectrum contained a very intense ion at m/e 292 (100%) and a small ion (3%) at m/e 368, the molecular weight of 17α-phenyl-5α-androstane-3β,17-diol[134]. Consequently it would appear that the desired product was present in this final fraction and the low intensity of its molecular ion in the mass spectrum may have been due to an intense fragmentation or, more probably, to masking by a minor, more volatile component of the mixture.

The explanation for the formation of diphenylcarbinol [138] and D-homo-3β-hydroxy-17aβ-methyl-17a-phenyl-18-nor-androst-13(14)-en-17-one[139] among the products of the reaction of epiandrosterone [135] and phenylmagnesium bromide [136] is at present unknown. The origin of the additional carbon atom is obviously a key question; consequently the purity of the bromobenzene used in the above reaction and in a repeat reaction, in which the same product mixture was obtained, was ascertained by refractometry. Since the steroidal starting material was pure attention must focus on the solvents, sodium-dried ether and tetrahydrofuran,

which was refluxed over and distilled from lithium aluminium hydride.[112g] Further experiments will be necessary to determine whether the tetrahydrofuran was the source of the extra carbon atom.

A reaction[117j] which leads to a product analogous to a compound which might be considered as a precursor of the D-homo-17aβ-methyl-17a-phenyl-17-ketone[139] is the D-homo rearrangement of a 17β-hydroxy-20-ketone of the pregnane series [140]. Lewis acid-catalysed rearrangement of such a compound leads to a D-homo-17aβ-hydroxy-17a-methyl-17-ketone[141] and the alkaline-catalysed reaction to the 17a-epimeric product [142]. By analogy, the rearrangement of a compound in which the 21-methyl group was replaced by a phenyl ring might lead to a D-homo-17a-hydroxy-17a-phenyl-ketone, the acid-catalysed dehydration of which might occur with migration of the C-18 methyl group to C-17a and formation of a $\Delta^{13(14)}$-double bond. However the origin of any intermediate containing the additional carbon atom is unknown, although it must surely be linked to the formation of diphenylcarbinol.

3.16. Experimental section.

3.16.i. 17β,20-Oxido-21-norpregn-5-en-3β-ol[125][190]

A solution of potassium t-butoxide (0.4g) in dimethyl sulphoxide (3ml) was added dropwise to a stirred, water-cooled solution of 3β-hydroxyandrost-5-en-17-one (500.3mg) and trimethylsulphonium iodide (474.8mg) in dimethyl sulphoxide (5ml) under nitrogen. After 2½ hours the flask was cooled during the slow addition of water, the steroid was extracted into ether, washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield white crystalline 17β,20-oxido-21-norpregn-5-en-3β-ol (478.8mg); m.p. 162-165° (lit.,[191] 172-176°); t.l.c., one spot, slightly less polar than the starting material; n.m.r. (100 M Hz) τ9.12 (C-18 methyl), 8.99 (C-19 methyl), 7.40, 7.10 (d,d,6 Hz, 6 Hz, $-OC\underline{H}_2$), 6.48 (m, $w_{\frac{1}{2}}$ 25 Hz, 3α-H), 4.64 (6-H); mass spectrum, M^+, m/e 302 (160°C).

3.16.ii. 17β,20-Oxido-21-norpregn-4-en-3-one[191]

17β,20-Oxido-21-norpregn-5-en-3β-ol (146.9mg) was dissolved in toluene (12ml) and cyclohexanone (2ml). Toluene (2ml) was removed by distillation prior to the addition of aluminium isopropoxide (293.8mg) in toluene (10ml). The mixture was refluxed gently for 3 hours, water was added and steam distillation carried out with a Soxhlet extractor for 1½ hours. The aqueous mixture in the reaction flask was extracted with chloroform, washed with very dilute hydrochloric acid, saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness. The product, an oil, smelled strongly

of cyclohexanone. Prolonged evaporation *in vacuo* and with a stream of nitrogen produced crystals which were filtered and washed with acetone to give a product (39.0mg, *ca.* 25%); n.m.r. (100 M Hz) showed that the Δ^5-en-3β-ol system had been oxidised to the Δ^4-en-3-one, τ4.26(4-H) but that the epoxide function had been degraded; the doublets at τ7.40, 7.10 (6 Hz) were absent.

3.16.iii. *Reaction of 17β,20-oxido-21-norpregn-5-en-3β-ol with boron trifluoride*

a. *In acetamide*

Melted acetamide (10g) was dried over molecular sieve and added to 17β,20-oxido-21-norpregn-5-en-3β-ol (103.3mg). The mixture was warmed until a solution was obtained whereupon redistilled boron trifluoride-etherate[194] (0.04ml) was added and the mixture heated at *ca.* 100° for 1¾ hours. Water was added and the steroid extracted into ether, washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a crude product (122.7mg); n.m.r. (100 M Hz) τ6.70, 6.60 (d, d, J_{AB} 10 Hz, $-C\underline{H}_2OH$), 5.83, 5.73 (d, d, J_{AB} 11 Hz, $-C\underline{H}_2OH$), containing approximately equal amounts of 17α-hydroxymethyl-17-methyl-18-norandrosta-5,13(14)-dien-3β-ol and 17α-hydroxymethylandrost-5-ene-3β,17-diol,[192] respectively. Crystallisation from acetone gave pure 17α-hydroxymethyl-17-methyl-18-norandrosta-5,13(14)-dien-3β-ol (18.5mg); m.p. 167-169°; t.l.c., one spot; n.m.r. (100 M Hz), τ9.02 (C-19 *methyl*), 9.02 (C-20 *methyl*), 6.70, 6.60 (d, d, J_{AB} 10 Hz, $-C\underline{H}_2OH$), 6.46 (m, 3α-H), 4.59 (6-H).

b. In benzene[195]

Redistilled boron trifluoride-etherate[194] (0.04ml) was added to a solution of 17β,20-oxido-21-norpregn-5-en-3β-ol (96.7mg) in anhydrous benzene (3ml). After 24 hours at room temperature ether was added. The solution was washed with sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a red glass (97.5mg). T.l.c. indicated that the product contained at least five components and the n.m.r. spectrum showed that much degradation had occurred. Peaks at τ9.02 (C-19 methyl), 9.02 (C-20 methyl), 6.65 ($-CH_2OH$) showed that 17α-hydroxymethyl-17-methyl-18-norandrosta-5,13(14)-dien-3β-ol was formed in the reaction. Because of the degradation which had occurred the product was not further studied.

3.16.iv. Reaction of 17α,20-oxido-21-norpregn-4-en-3-one with boron trifluoride

a. In acetamide

17α,20-Oxido-21-norpregn-4-en-3-one (96.3mg) was reacted with boron trifluoride-etherate[194] (0.04ml) in acetamide (10g) as described in section 3.16.iiia to give 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one (100.7mg) as the major product, identified by its n.m.r. spectrum (100 M Hz); τ9.03 (C-20 methyl), 8.86 (C-19 methyl), 6.70, 6.60 (d, d, J_{AB} 10 Hz, $-C\underline{H}_2OH$), 4.26 (4-H).

b. In benzene[195]

17α,20-Oxido-21-norpregn-4-en-3-one (98.6mg) was

reacted with boron trifluoride-etherate[194] (0.04ml) in benzene (3ml) as described in section 3.16.iiib. The product, a red glass (100.3mg), was a complicated mixture which contained 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one; n.m.r. (100 M Hz) τ 6.70, 6.60 (d, d, J_{AB} 10 Hz, $-C\underline{H}_2OH$) and many products of degradation which were not further studied.

The reaction was repeated, but allowed to proceed for a much shorter time. 17α,20-Oxido-21-norpregn-4-en-3-one (143mg) was dissolved in dry benzene (14.3ml) and boron trifluoride-etherate (2 drops) was added to the stirred solution. After 2 minutes the reaction mixture was poured into ether and water, the ether solution was washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a yellow glass (157.2mg). T.l.c. and n.m.r. spectroscopy (100 M Hz) showed that the major product was 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one, τ9.02 (C-20 methyl), 8.85 (C-19 methyl), 6.70, 6.60 (d, d, J_{AB} 10 Hz, $-C\underline{H}_2OH$), 4.27 (4-H).

3.16.v. Reaction of 17α-hydroxy-17-hydroxymethylandrost-4-en-3-one with sulphuric acid

A mixture of 17α-hydroxy-17-hydroxymethylandrost-4-en-3-one (34.1mg, prepared in section 3.2.v) and 20% aqueous sulphuric acid (6ml) was heated at 100° for 2 hours. When cool, ether was added and the organic extract was washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a red glass (43.1mg); t.l.c. showed a

complicated mixture; n.m.r. (100 M Hz) showed that the major product was 17α-hydroxymethyl-17-methyl-18-norandrosta-4,13(14)-dien-3-one, τ9.01 (C-20 methyl), 8.84 (C-19 methyl), 6.70, 6.60 (d, d, J_{AB} 10 Hz, $-C\underline{H}_2OH$), 4.25 (4-H).

3.16.vi. Attempted synthesis of 17α-phenyl-5α-androstane-3β,17-diol

A portion of a mixture of bromobenzene (5.42g, 3.61ml) and ether (20ml) was added to dry magnesium turnings (0.83g) in ether (5ml). A crystal of iodine was added and gentle heat supplied until turbidity was observed. The remainder of the bromobenzene solution was then slowly added and the mixture refluxed for 15 minutes. A solution of epiandrosterone (1.003g) in tetrahydrofuran (50ml) was added dropwise to the solution of Grignard reagent with swirling. A white precipitate which formed on addition of the steroid solution dissolved on mixing and considerable heat was generated during the process. During removal of ether (10ml) by distillation of the brown solution precipitation occurred. The white precipitate and yellow liquid were stirred and refluxed for 17½ hours. The reaction mixture was then poured into a mixture of ice, water and concentrated sulphuric acid (4ml). Ether was added and the aqueous layer reextracted with ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a viscous orange oil (3.05g). T.l.c. showed that the crude product contained at least six components. Gradual crystallisation occurred on standing

but crystals could not be obtained by filtration. Consequently the crude product was dissolved in benzene and chromatographed on 5% deactivated alumina (90g).

Elution with petrol (60-80°) and with benzene-petrol (1:1) gave a mixture (0.2g) containing two components of low polarity and low melting points (40.5-63°). The major component of the fraction was identified as diphenyl from its infrared and n.m.r. spectra; Mass spectrum, M^+, m/e 154.

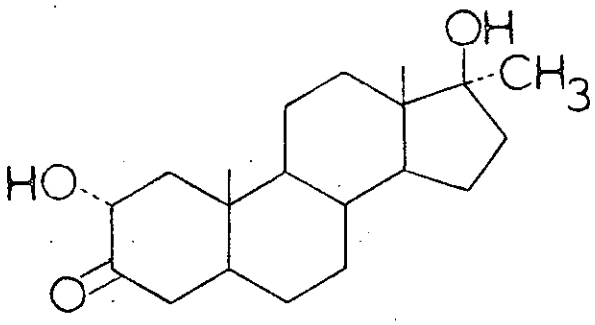
Continued elution with benzene-petrol (1:1) gave a single component (1.2g), identical with the more polar component of the previous fraction and identified as diphenylcarbinol; m.p. 62.5-63.5° (lit.,[193] 69°); ν_{max}3650cm^{-1} and aromatic bands; n.m.r. (100 M Hz) τ7.59 (s, 1H, C<u>H</u>), 4.23 (s, 1H, -O<u>H</u>), 2.69 (s, 10 H, Ar<u>H</u>); red solution with concentrated sulphuric acid;[193] Mass spectrum, M^+, m/e 184.

Elution with benzene and then with ethanol gave material (0.88g) which was shown, by t.l.c., to contain a mixture of several components. Crystallisation of this fraction from acetone gave a single compound (93mg), the only component of the crude fraction visible under ultraviolet light; t.l.c., R_f 0.46 (<u>cf</u>. epiandrosterone, R_f 0.41); m.p. 175.5-177.5°; ν_{max}3350,2950,1720,1635,1050,780,700cm^{-1}; n.m.r. (100 M Hz) τ9.13 (C-19 <u>methyl</u>), 9.05 (C-17a <u>methyl</u>), 7.75-7.00 (7 doublets, 2 H, C-16 <u>H</u>), 6.40 (m, 3α-<u>H</u>), 2.70-2.40 (m, 5 H, Ar<u>H</u>); Mass spectrum, M^+, m/e 378; calculated for $C_{26}H_{34}O_2$ 378.255866, found 378.255759. This product was thus tentatively identified as D-homo-3β-hydroxy-17aβ-methyl-17a-phenyl-18-norandrost-13(14)-en-17-one which

structure was consistent with the data.

The column was stripped by further elution with ethanol to give a mixture (0.08g) containing several polar components, one of which was visible under ultraviolet light; ν_{max} 3450, 2950, 710 cm^{-1}; n.m.r. (100 M Hz) τ9.24 (C-18 methyl), 9.18 (C-19 methyl), 6.38 (m, 3α-H), 2.68 (Ar H); Mass spectrum, intense ion at m/e 292 (100%) and ion at m/e 368 (3%, M^+ of 17α-phenyl-5α-androstane-3β,17-diol). This fraction may therefore have contained 17α-phenyl-5α-androstane-3β,17-diol.

The major product of the above reaction, assumed to be D-homo-3β-hydroxy-17aβ-methyl-17a-phenyl-18-norandrost-13(14)-en-17-one was acetylated with acetic anhydride and pyridine at room temperature for $2\frac{1}{2}$ hours to give the 3β-acetate; m.p. 226-228°; ν_{max} 1730, 1720 cm^{-1}; n.m.r. (100 M Hz) τ9.12 (C-19 methyl), 9.06 (C-17a methyl), 8.00 ($COCH_3$), 5.33 (m, 3α-H), 2.70-2.40 (Ar H); Mass spectrum, M^+, m/e 420.



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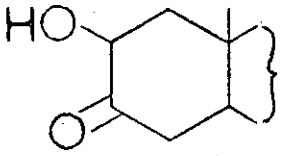

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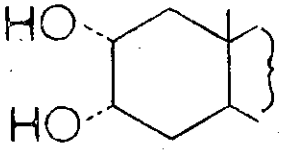

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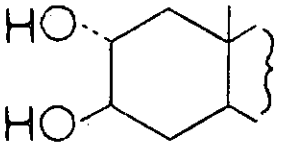

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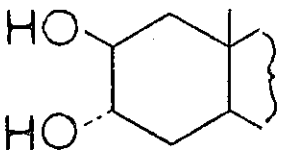

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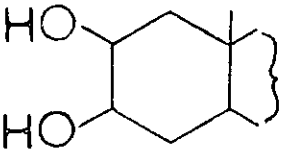


[148]

4.1. The synthesis of the 17α-methyl-5α-androstane-2,3,17-triols as standards for the metabolites of oxymetholone

4.1.i. Following the administration of oxymetholone to normal men Adhikary[32] isolated two major metabolites, both of which were more polar than the drug. High temperature catalytic reduction of all three compounds gave products with the same retention times as those obtained from steroids with the 17-methylandrostane skeleton. Consequently it could not be determined whether the C-2 substituent in oxymetholone was removed during metabolism or retained in the metabolites and removed during carbon skeleton chromatography.

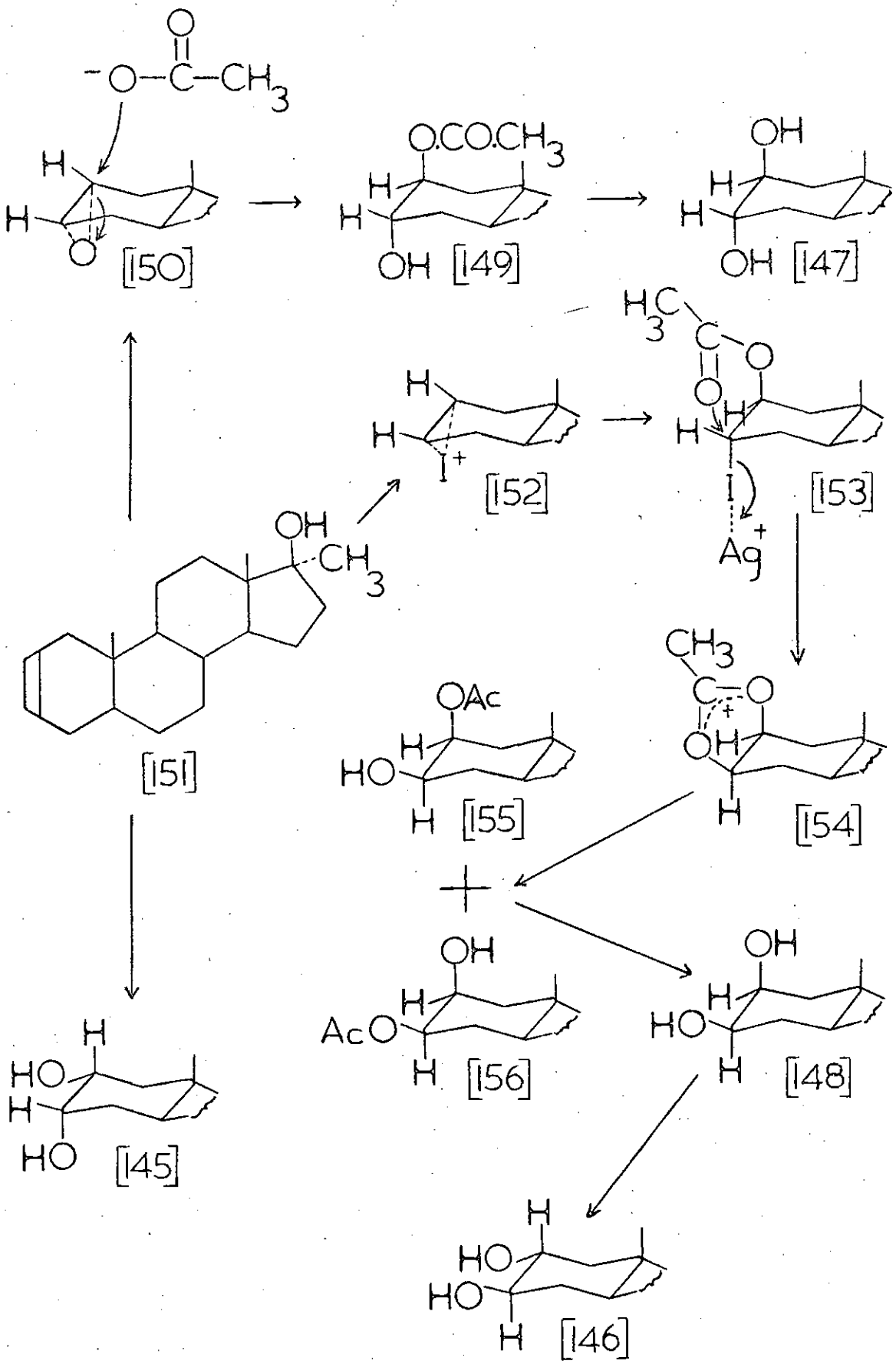
Adhikary[32] reported that the more polar of the two metabolites (O-1) was not reduced with potassium borohydride but that the less polar metabolite (O-2) was reduced to a product with the same polarity as O-1. He thus suggested[32] that O-2 might contain a 2-hydroxy-3-keto function [143,144] and O-1 a 2,3-glycol system [145-148]. Because of the polar nature of the metabolites of oxymetholone purification was difficult;[32] consequently the synthesis of the six 17β-hydroxy-17-methyl-5α-androstanes containing the above substituents [143-148] was attempted to provide compounds for comparison with the metabolic extracts.

4.1.ii. 17α-Methyl-5α-androst-2-en-17-ol

The most convenient intermediate for the synthesis of the 2,3-dioxygenated compounds [143-148] was 17α-methyl-5α-androst-2-en-17-ol, which was prepared by a route starting from dehydroepiandrosterone. Hydrogenation of this compound with a palladium-charcoal catalyst by the method of

Pettit *et al*[196] gave 3β-hydroxy-5α-androstan-17-one as the major product. Alumina chromatography of the crude reduction product gave a low polarity material (5.3%) which was identified as a mixture of 5α-androstan-17-one, τ9.17 (C-19 methyl), 9.12 (C-18 methyl), (lit.,[110] τ9.19, 9.14) with a small amount of 5β-androstan-17-one, τ9.05 (C-19 methyl), 9.12 (C-18 methyl), (lit.,[110] τ9.05, 9.15). It has been reported[196] that hydrogenolysis (*ca*. 5%) of the C-3 function generally accompanies catalytic reduction of Δ^5-en-3β-ols.

3β-Hydroxy-5α-androstan-17-one tosylate was prepared by the standard procedure[112c] and adsorbed onto alumina in an attempt to obtain the Δ^2-ene. Although the synthesis of 5α-cholest-2-ene in 70% yield by the reaction of the 3β-tosylate on alumina has been reported,[197] under similar conditions 5α-androst-2-en-17-one was obtained in only 6.5% yield. Iriarte *et al*.[198] have, however, examined the reaction of 3β-hydroxy-5α-androstan-17-one tosylate with sodium acetate in acetic anhydride and acetic acid as a means of preparing 3α-hydroxy-5α-androstan-17-one and obtained 5α-androst-2-en-17-one in 54% yield together with 3α-hydroxy-5α-androstan-17-one acetate (39%). Consequently, 5α-androst-2-en-17-one was prepared by this method and reacted with a ten-fold excess of methylmagnesium iodide to give the known 17α-methyl-5α-androst-2-en-17-ol[199] in 61% yield.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4.1.iii. 17α-Methyl-5α-androstane-2α,3α,17-, 2α,3β,17-, 2β,3α,17- and 2β,3β,17-triol[145-148].

Procedures for the stereospecific synthesis of the four steroid 2,3-glycols were devised many years ago and are well established. In 1957, Henbest and Smith[200] prepared the four cholestane-2,3-diols, the 2α,3α-, 2β,3β- and 2α,3β-isomers for the first time, by methods similar to those used for the synthesis of corresponding diols in the sapogenin series. The configurations in the cholestanes were assigned by analogy with the sapogenin series.

Marker and Plambeck[201] prepared the 2β,3α-diol in the cholestane series by the action of hydrogen peroxide on the Δ^2-ene. During the synthesis of a variety of 2,3-oxygenated-17α-methyl-5α-androstan-17-ols Klimstra[202] prepared 17α-methyl-5α-androstane-2β,3α,17-triol 2-acetate[149] by the acetic acid cleavage of the 2α,3α-epoxide[150] obtained from the 2-ene[151]. Consequently 17α-methyl-5α-androstane-2β,3α,17-triol[147] was prepared by hydrolysis of the 2β-acetate[149] obtained by the above route.

17α-Methyl-5α-androstane-2β,3β,17-triol[148] was prepared by the reaction of iodine and silver acetate with 17α-methyl-5α-androst-2-en-17-ol[151]. The reagents serve as a source of positive iodine which reacts[117k,203] with the olefin [151] to form the 2α,3α-iodinium ion [152]. This species is attacked by the acetate ion to form an iodo-acetate [153] which is converted to the 2β,3β-acetonium bridged ion [154] by Ag^+-catalysed loss of the iodide ion in anhydrous acetic acid. The addition of water to the acetonium ion [154] leads to rapid formation of the two mono-

acetates of the 2β,3β-diol [155,156] which are then hydrolysed[117k] to the free glycol [147].

17α-Methyl-5α-androstane-2α,3β,17-triol[146] was prepared by epimerisation of the axial 2β-hydroxyl group in the corresponding 2β,3β-glycol[148] to the more stable equatorial position with sodium in ethanol.[200] The reaction[1171,200] of 17α-methyl-5α-androst-2-en-17-ol[151] with osmic acid was used for the preparation of the cis 2α,3α-glycol [145].

The n.m.r. spectra of the four triols [145-148] were obtained in deuteriochloroform solutions although their solubility was poor, so that the C-18 and C-19 methyl positions could be compared with the theoretical values computed from tables of substituent shifts[110,204a] [table 11]. The agreement between the theoretical and observed positions was good except for the 2β,3β-glycol. Other characteristics of the compounds are described in section 4.3.

4.1.iv. 2β,17β-Dihydroxy-17-methyl-5α-androstan-3-one 2-acetate

Klimstra[202] prepared 2β,17β-dihydroxy-17-methyl-5α-androstan-3-one 2-acetate by chromic acid oxidation of the 2β-acetoxy-3α-ol. This reaction was carried out a number of times in an attempt to prepare the 2-hydroxy-3-ketone by hydrolysis of the 2-acetoxy-3-one but in each case the product of the oxidation reaction was an intractable gum. In the light of results described in section 4.3 attempts to prepare this compound were not continued.

τ ($CDCl_3$)	2α, 3α		2α, 3β		2β, 3α		2β, 3β	
	19-H	18-H	19-H	18-H	19-H	18-H	19-H	18-H
5α,14α-[110]	9.22	9.31	9.22	9.31	9.22	9.31	9.22	9.31
17β-OH,17-Me[204a]	-0.01	-0.15	-0.01	-0.15	-0.01	-0.15	-0.01	-0.15
2α-OH[110]	-0.02	0.00	-0.02	0.00	-	-	-	-
2β-OH[110]	-	-	-	-	-0.26	-0.01	-0.26	-0.01
3α-OH[110]	0.00	-0.01	-	-	0.00	-0.01	-	-
3β-OH[110]	-	-	-0.03	-0.01	-	-	-0.03	-0.01
Calculated	9.19	9.15	9.16	9.15	8.95	9.14	8.92	9.14
Found	9.19	9.17	9.17	9.17	8.97	9.14	9.00	9.18

Table 11. The theoretical and observed τ values of the C-18 and C-19 methyl resonances in the four 17α-methyl-5α-androstane-2,3,17-triols.

4.2. Experimental Section

4.2.i. 3β-Hydroxy-5α-androstan-17-one[196]

10% Palladium on charcoal (2.30g) and 72% perchloric acid (0.2ml) were added to a solution of 3β-hydroxyandrost-5-en-17-one (20g) in absolute ethanol (250ml) and hydrogenated. The reaction was continued for 4 hours although 90% of the hydrogen was consumed within the first hour. The solution was filtered through a layer of Celite and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in chloroform, washed with water, saturated sodium bicarbonate solution and with water again, dried over anhydrous magnesium sulphate and evaporated to dryness to give a product (19.01g) containing two components. The major component was of similar polarity to the starting material and the other of lower polarity. The product was chromatographed on alumina (350g) and the low polarity fraction (1.51g, 5.3%) eluted with benzene and with benzene-chloroform (4:1); n.m.r. (60 M Hz) τ9.17 (C-19 methyl), 9.12 (C-18 methyl), 5α-androstan-17-one (lit.,[110] τ9.19, 9.14); and τ9.12 (C-18 methyl), 9.05 (C-19 methyl), 5β-androstan-17-one (lit.,[110] τ9.15, 9.05). The polar product (15.21g) was eluted with chloroform and crystallised from chloroform-hexane (1:2) to give pure 3β-hydroxy-5α-androstan-17-one (14.7g, 73%); m.p. 173-174.5° (lit., [196] 176-177.5°); ν_{max} 3450,2930,1730,1380,1045cm^{-1}; n.m.r. (100 M Hz) τ9.17 (C-19 methyl), 9.15 (C-18 methyl), 6.41 (sp, 10,10,5,5 Hz, 3α-H).

4.2.ii. 3β-Hydroxy-5α-androstan-17-one tosylate[112c]

A solution of freshly purified toluene-p-sulphonyl chloride[112c] (20g) in dry pyridine (100ml) at 0° was added to a solution of 3β-hydroxy-5α-androstan-17-one (14.7g) in pyridine (200ml) also at 0°. The mixture was maintained at 0° for 48 hours when the solution was a deep red-orange colour, indicative of formation of the derivative.[112c] The solution was poured into an ice-water mixture and stirred until all the ice had melted. The precipitated tosylate was recovered by filtration, washed with very dilute hydrochloric acid and with water and dried in vacuo to give 3β-hydroxy-5α-androstan-17-one tosylate (20.71g, 92%); t.l.c., one spot of low polarity in comparison to the free alcohol; m.p. 160-161° (lit.,[198] 163-164°); ν_{max} 2920,1735,1380,1195,1180cm^{-1}; n.m.r. (60 M Hz) τ9.21 (C-19 methyl), 9.18 (C-18 methyl), 7.57 (Ar-C$\underline{H}_3$), 5.56 (m, 3α-$\underline{H}$), 2.71, 2.23 (d, d, 8.4, 8.4 Hz, 4H, Ar-$\underline{H}$).

4.2.iii. 5α-Androst-2-en-17-one

a. Reaction of 3β-hydroxy-5α-androstan-17-one tosylate with alumina[197]

3β-Hydroxy-5α-androstan-17-one tosylate (5.93g) was dissolved in a small volume of benzene and adsorbed onto a column of alumina (200g). After 36 hours the column was eluted with benzene to give 5α-androst-2-en-17-one (0.39g, 6.5%); ν_{max} 3000,1735,1655,675cm^{-1}; n.m.r. (60 M Hz) τ9.23 (C-19 methyl), 9.14 (C-18 methyl), 4.40 (d, 1.2 Hz, 2-$\underline{H}$, 3-$\underline{H}$). Elution with ethanol gave a compound (4.87g, 82%); ν_{max} 3450, 1735,1380,1045,1035,1020cm^{-1}; n.m.r. (60 M Hz) τ9.17 (C-19

methyl), 9.15 (C-18 methyl), 6.41 (m, 3α-H) which was thus identified as 3β-hydroxy-5α-androstan-17-one rather than the 3α-hydroxy epimer(lit.,[110] 3β-H, τ5.9-6.0).

b. Reaction of 3β-hydroxy-5α-androstan-17-one tosylate with sodium acetate in acetic anhydride[198]

A solution of 3β-hydroxy-5α-androstan-17-one tosylate (19.52g) and anhydrous sodium acetate (19.52g) in glacial acetic acid (173.6ml) and acetic anhydride (17.36ml) was refluxed for 5 hours. Chloroform and water were added, the layers were separated and the aqueous layer reextracted with chloroform. The combined organic layers were washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness. The residue was chromatographed on silica gel (750g) and elution with benzene (4l) gave 5α-androst-2-en-17-one (6.91g, 57%) as crystalline plates; m.p. 99-100.5° (lit.,[198] 105-106°); ν_{max}3000,1735,1655,675cm^{-1}; n.m.r. (60 M Hz) τ9.24 (C-19 methyl), 9.15 (C-18 methyl), 4.41 (d, 1.2 Hz, 2-H, 3-H). Elution with benzene-ether (2:1,11)gave 3α-hydroxy-5α-androstan-17-one acetate (5.77g, 39%); m.p. 163-164° (lit.,[198] 164-165°); ν_{max}1735cm^{-1}; n.m.r. (60 M Hz) τ9.19 (C-19 methyl), 9.15 (C-18 methyl), 7.96 ($COCH_3$), 4.99 (m, 3β-H).

4.2.iv. 17α-Methyl-5α-androst-2-en-17-ol[151]

A solution of methyl iodide (34.1g, 15ml) in dry ether (120ml) was added to magnesium turnings (5.76g) in ether (40ml) and refluxed for 15 minutes to give methyl-magnesium iodide. A solution of 5α-androst-2-en-17-one

(6.53g) in ether (200 ml) was added dropwise to the cooled Grignard reagent and refluxed for 15 minutes. It was then poured into a mixture of ice, concentrated sulphuric acid and water and stirred until the ice had melted. The layers were separated, the aqueous layer extracted with ether and the combined organic extracts washed with dilute hydrochloric acid, sodium metabisulphite solution and with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness to give a yellow glass (6.835g) which was chromatographed on alumina (240g). Unreacted 5α-androst-2-en-17-one (2.52g) was eluted with benzene and identified by its n.m.r. and infrared spectra. Elution with benzene-chloroform (1:1) gave 17α-methyl-5α-androst-2-en-17-ol (4.22g, 61%); m.p. 144-146° (lit.,[199] 147°); n.m.r. (100 M Hz) τ9.23 (C-19 methyl), 9.15 (C-18 methyl), 8.80 (C-20 methyl), 4.42 (d, 2 Hz, 2-H, 3-H).

4.2.v. 2α,3α-Oxido-17α-methyl-5α-androstan-17-ol[150][205]

A solution of m-chloroperbenzoic acid (1.461g) in ether (40ml) was added to a solution of 17α-methyl-5α-androst-2-en-17-ol (0.992g) in ether (100ml). After 48 hours at room temperature the excess peracid was destroyed with sodium sulphite solution and ether was added. The organic phase was washed twice with sodium bicarbonate solution and then with water, dried over anhydrous magnesium sulphate and evaporated to dryness. Crystallisation from methanol gave 2α,3α-oxido-17α-methyl-5α-androstan-17-ol (0.635g, 60%) as needles; m.p. 197-200° (lit.,[202] 205-207°); n.m.r. (100 M Hz) τ9.24 (C-19 methyl), 9.17 (C-18 methyl), 8.81 (C-20

methyl), 6.86 (2β-H, 3β-H).

4.2.vi. 17α-Methyl-5α-androstane-2β,3α,17-triol 2-acetate [149][202]

2α,3α-Oxido-17α-methyl-5α-androstan-17-ol (0.4g) was dissolved in glacial acetic acid (8ml) and heated at 100° for 6 hours. When cool, ether and water were added, the ether extract was washed with saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give 17α-methyl-5α-androstane-2β,3α,17-triol 2-acetate; n.m.r. (60 M Hz) τ9.15 (C-18 methyl), 9.07 (C-19 methyl), 8.79 (C-20 methyl), 7.96 ($COCH_3$), 6.13 (m, 3β-H), 5.10 (m, 2α-H); ν_{max} 1710cm^{-1}. The product was partially purified by alumina chromatography.

4.2.vii. 17α-Methyl-5α-androstane-2β,3α,17-triol[147]

The product of the previous reaction, eluted from alumina with benzene-ethanol (19:1) was dissolved in methanol (1ml) with potassium hydroxide (0.15g). After 4 hours at room temperature the methanol was removed by evaporation *in vacuo* and the residue was dissolved in ether. The solution was washed with dilute hydrochloric acid, sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a crude product; m.p. 200-207°. Crystallisation from acetone gave 17α-methyl-5α-androstane-2β,3α,17-triol (0.15g); m.p. 217-218°; n.m.r. (60 M Hz) τ9.14 (C-18 methyl), 8.97 (C-19 methyl), 8.78 (C-20 methyl), 6.33 (m, 3β-H), 6.12 (m, 2α-H); Mass spectrum, M^+, m/e 322; calculated for $C_{20}H_{34}O_3$ 322.250781, found 322.249746.

4.2.viii. 17α-Methyl-5α-androstane-2β,3β,17-triol[148][200]

Freshly prepared silver acetate (0.77g) and iodine (0.49g) were added to a stirred solution of 17α-methyl-5α-androst-2-en-17-ol (0.5g) in glacial acetic acid (100ml). When all the iodine had dissolved water (1 drop) was added and the mixture was stirred at 95° for 20 hours. Sodium chloride (1g) in water (1ml) was added to the cooled mixture which was then filtered and the filtrate evaporated to dryness. The residue was hydrolysed with potassium hydroxide (1.5g) in methanol (75ml) at room temperature for 12 hours. The methanol was removed by evaporation *in vacuo* and the residue dissolved in chloroform, washed with water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness. The crude product was dissolved in benzene and chromatographed on alumina. Material of relatively low polarity, eluted with ether-methanol (49:1) was discarded. Elution with ether-methanol (1:1) gave 17α-methyl-5α-androstane-2β,3β,17-triol (0.37g) which was crystallised from methanol to give needles; m.p. 201-202°; n.m.r. (60 M Hz) τ9.18(C-18 *methyl*), 9.00 (C-19 *methyl*), 8.82 (C-20 *methyl*), 6.37 (m, 3α-*H*), 5.99 (m, 2α-*H*); Mass spectrum, M^+, m/e 322; calculated for $C_{20}H_{34}O_3$ 322.250781, found 322.250656.

4.2.ix. 17α-Methyl-5α-androstane-2α,3β,17-triol[146][200]

17α-Methyl-5α-androstane-2β,3β,17-triol (50mg) was dissolved in ethanol (6ml) containing sodium (*ca.* 200mg) and refluxed for 5 hours. The ethanol was removed and the residue dissolved in ether, washed with sodium bicarbonate

solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give 17α-methyl-5α-androstane-2α,3β,17-triol which was crystallised from acetone; m.p. 190-192°; n.m.r. (60 M Hz) τ9.17 (C-19 methyl), 9.17 (C-18 methyl), 8.81 (C-20 methyl), 6.54 (m, 3α-H), 6.04 (m, 2β-H), Mass spectrum, M^+, m/e 322; calculated for $C_{20}H_{34}O_3$ 322.250781, found 322.249306; 17α-methyl-5α-androstane-2α,3β,17-triol 2,3-acetonide, M^+, m/e 362; calculated for $C_{23}H_{38}O_3$ 362.282079, found 362.282610; M-15, m/e 347, calculated for $C_{22}H_{35}O_3$ 347.258605, found 347.258286.

4.2.x. 17α-Methyl-5α-androstane-2α,3α,17-triol[145][200]

A solution of 17α-methyl-5α-androst-2-en-17-ol (0.5g) in dry benzene (15ml) was added to a solution of osmic acid (0.5g) in pyridine (25ml). After 5 days at room temperature the mixture was saturated with hydrogen sulphide, the solvents were removed by evaporation in vacuo and the residue was dissolved in benzene. The solution was filtered twice through Celite and the filtrate evaporated to dryness. Crystallisation from methanol gave 17α-methyl-5α-androstane-2α,3α,17-triol; m.p. 197-199°; n.m.r. (100 M Hz) τ9.19 (C-19 methyl), 9.17 (C-18 methyl), 8.81 (C-20 methyl), 6.27 (m, 2β-H), 6.06 (m, 3β-H); Mass spectrum, M^+, m/e 322; calculated for $C_{20}H_{34}O_3$ 322.250781, found 322.250342.

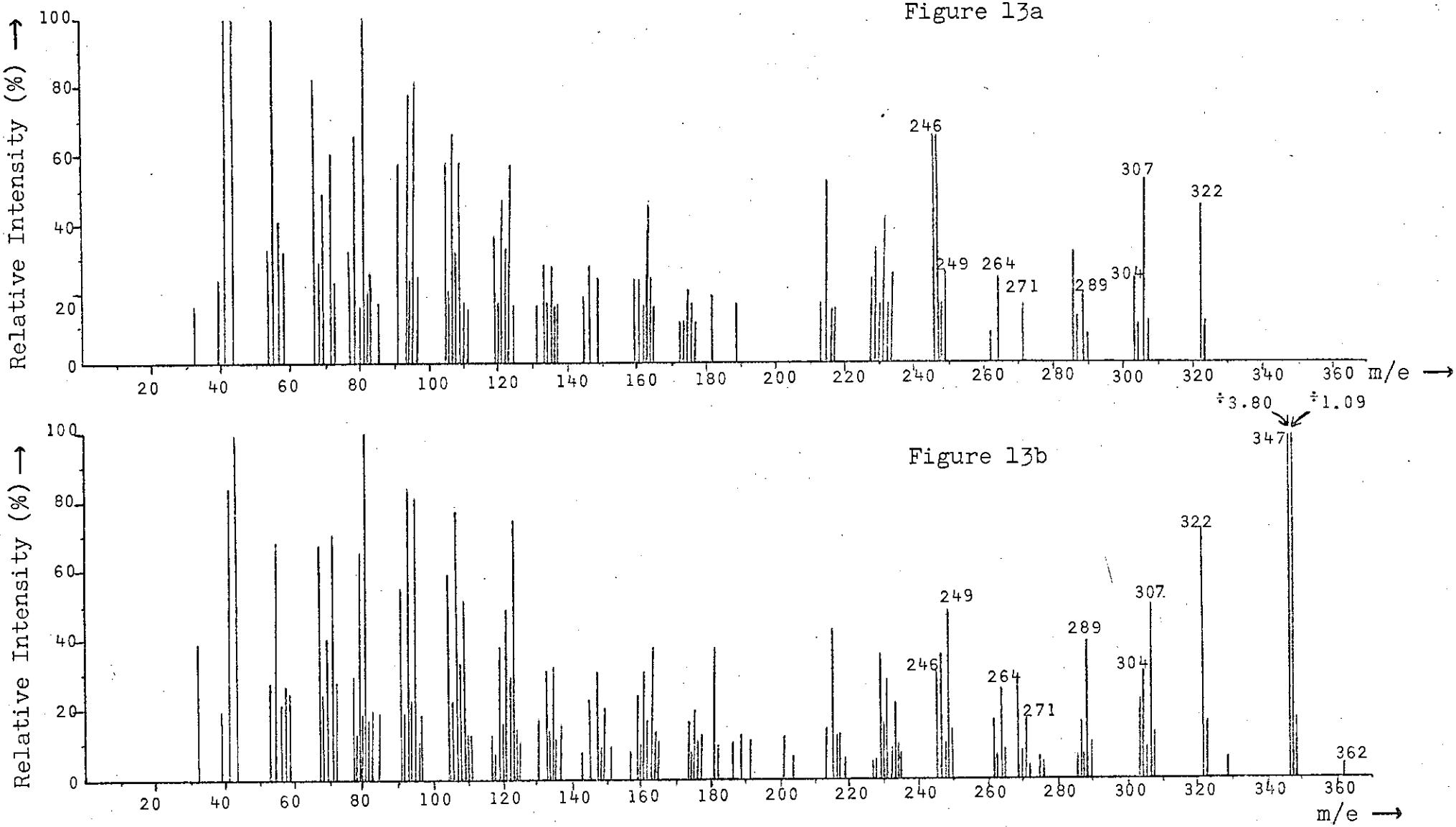


Figure 13. The mass spectra of a) 17α-methyl-5α-androstane-2α,3α,17-triol and b) 17α-methyl-5α-androstane-2α,3β,17-triol.

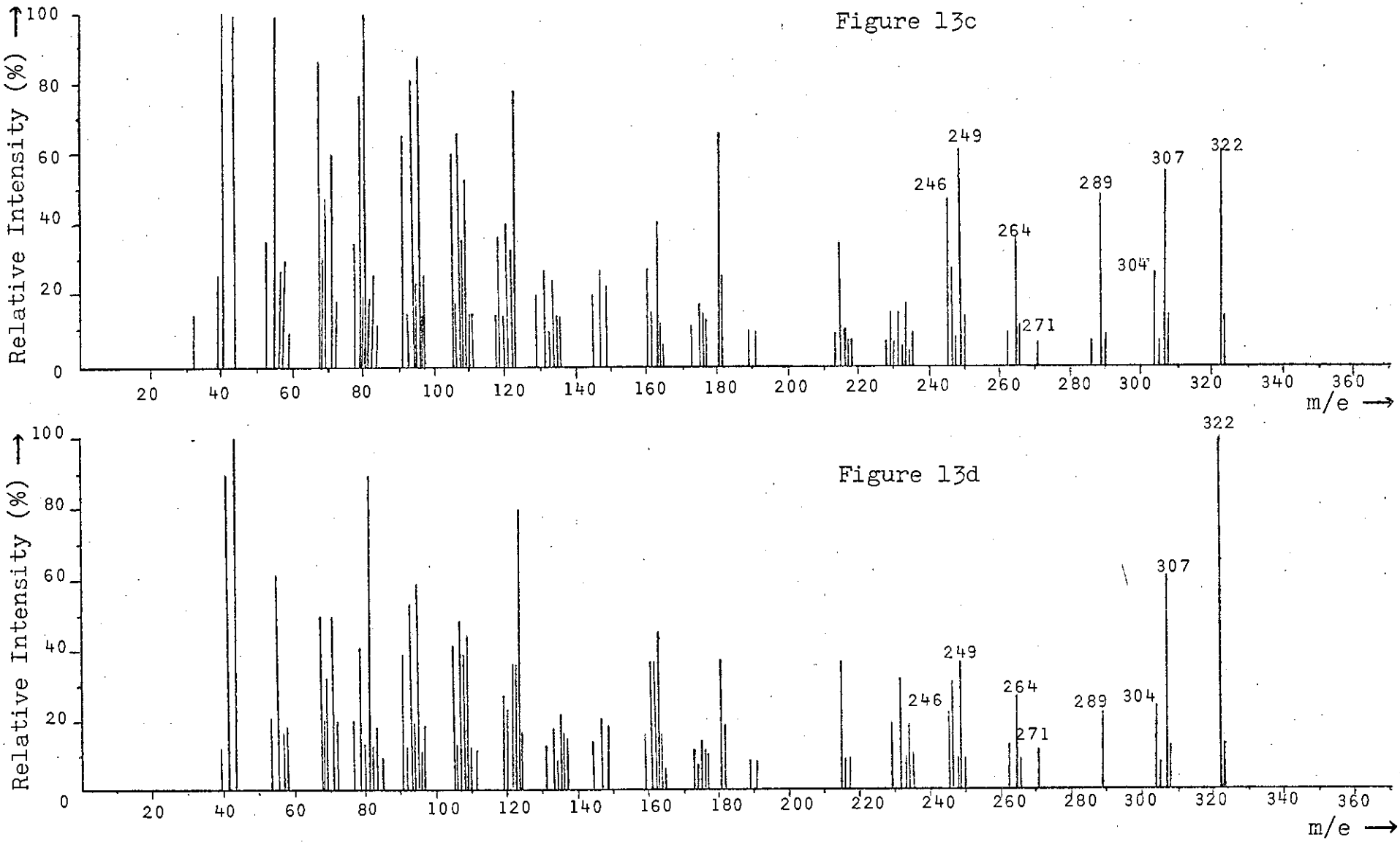


Figure 13. The mass spectra of c) 17α-methyl-5α-androstane-2β,3α,17-triol and d) 17α-methyl-5α-androstane-2β,3β,17-triol.

4.3. The comparison of the 17α-methyl-5α-androstane-2,3,17-triols with the metabolites of oxymetholone.

The four 17α-methyl-5α-androstane-2,3,17-triols were examined by t.l.c. in benzene-ethanol (9:1) and chloroform-methanol (9:1) solvent systems. When the plates were developed with sulphuric acid and heated the compounds gave yellow-brown spots of slightly differing polarities. The R_f values of the 2α,3α-, 2α,3β-, 2β,3α- and 2β,3β- compounds relative to that of oxymetholone expressed as 1.00 were 0.54, 0.41, 0.39 and 0.59 in the former solvent system and 0.70, 0.63, 0.54 and 0.68 in the latter. All were considerably more polar than oxymetholone although the 2α,3β-glycol appeared to contain a trace of material (relative R_f 1.07) of lower polarity than oxymetholone; this was not present in any of the other compounds. Comparison of the R_f values of the four triols and a sample of the more polar metabolite of oxymetholone showed that the metabolite (relative R_f 0.46 in $CHCl_3$-MeOH) was more polar than any of the standard triols. Since a 2,3-glycol is expected to be more polar than the corresponding 2-hydroxy-3-ketone doubt was thus cast upon the suggested[32] identities of O-1 and O-2.

The mass spectra of the four synthetic triols [figures 13a, 13b, 13c, 13d] were compared prior to examination of the spectra of metabolic samples. The 2α,3α-, 2β,3α- and 2β,3β-glycols [see figures 13a, 13c, 13d] gave the correct molecular ion for a 17α-methyl-5α-androstane-2,3,17-triol, at m/e 322. The most intense ions in the spectra of these epimers were those at low m/e values; in the high mass range each spectrum contained a series of intense ions at the same m/e

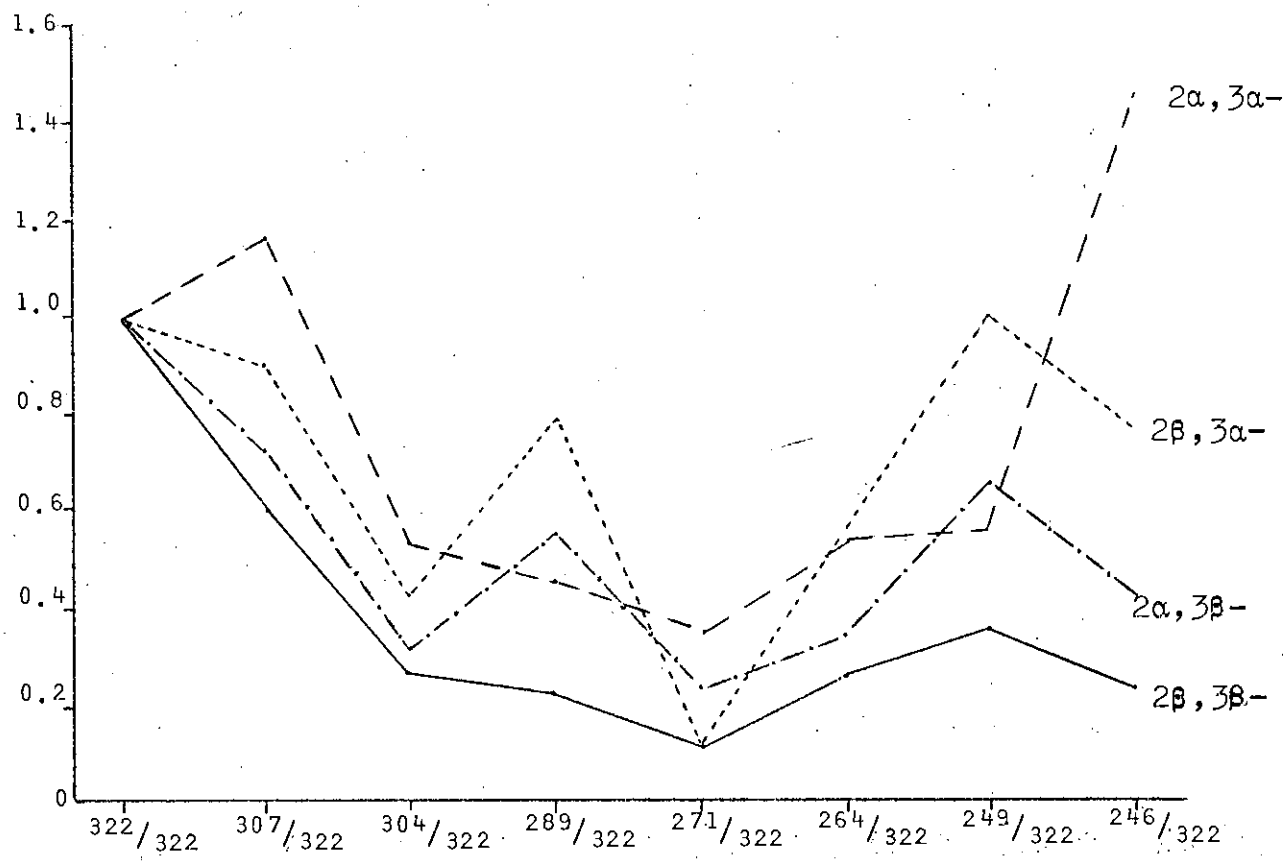


Figure 14. The relative intensities of characteristic ions in the mass spectra of the 17α-methyl-5α-androstane-2,3,17-triols.

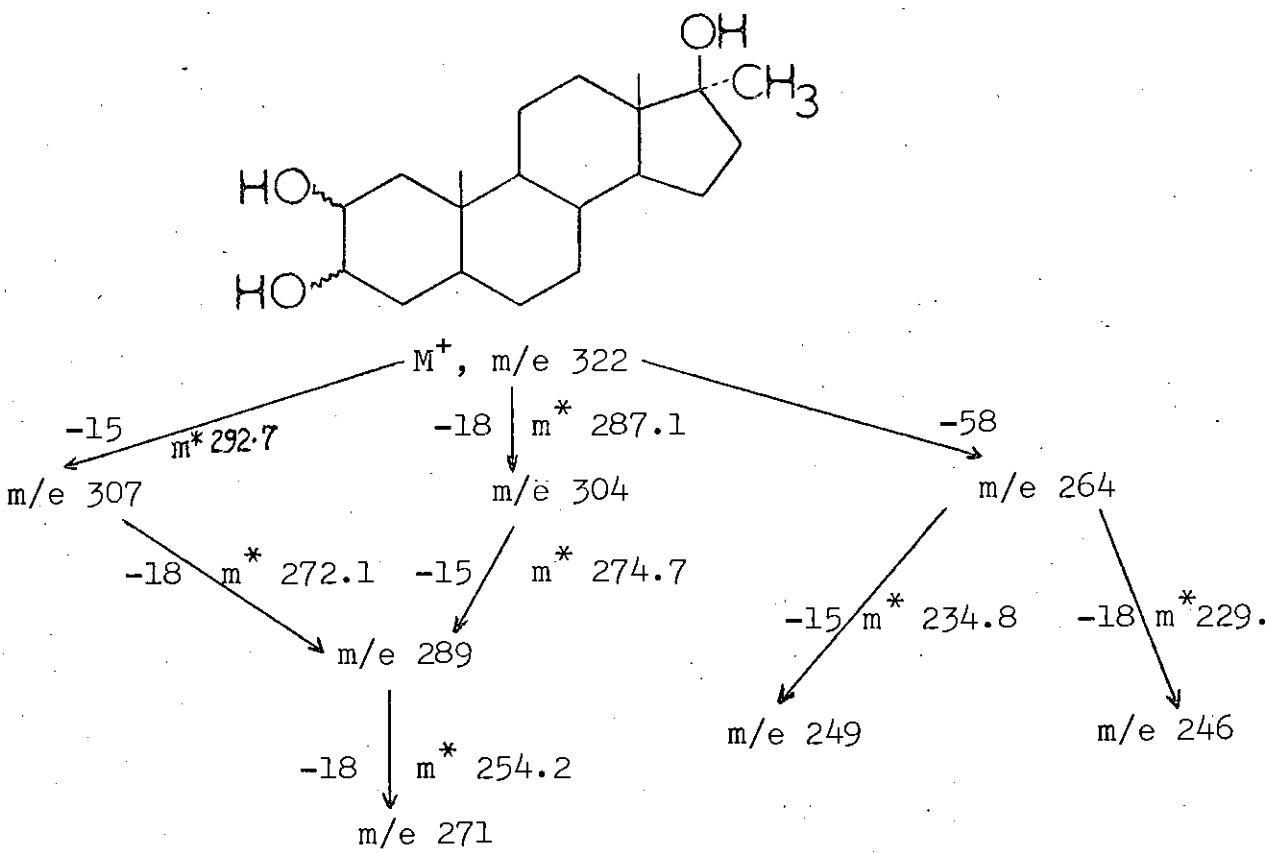
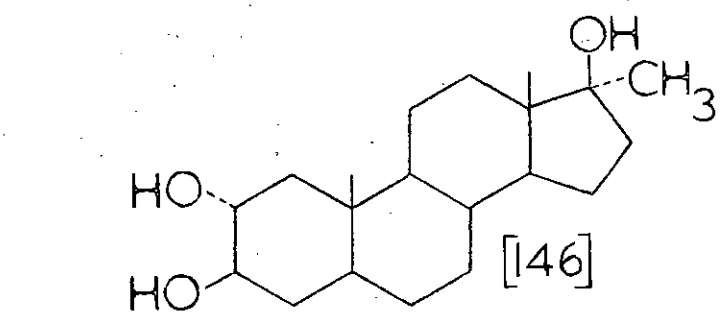


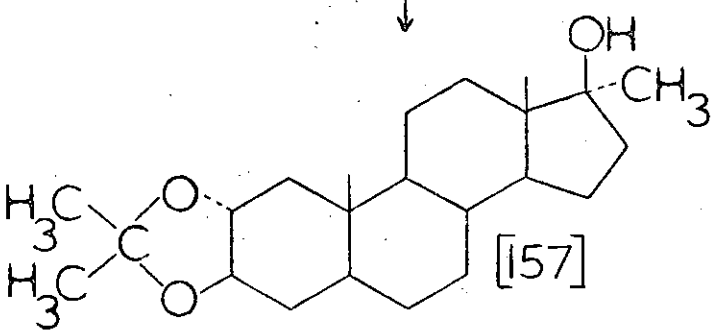
Diagram 18. Mass spectral fragmentations of 17α-methyl-5α-androstane-2,3,17-triols

values but of different intensities relative to one another [figure 14]. Metastable ions were available to show that these ions arose from loss of a methyl group (m/e 307) and of water (m/e 304) from M^+, loss of water from m/e 307 (m/e 289) and of a methyl group from m/e 304 (m/e 289) and of water from m/e 289 (m/e 271). An alternative fragmentation involved loss of an uncharged species of mass 58 from M^+ to give m/e 264 (D-ring fragmentation) which fragment then lost either water (m/e 246) or a methyl group (m/e 249) [Diagram 18]. The patterns of the relative intensities of these seven ions and M^+, shown in figure 14, were different for each epimer. The stability of the molecular ion of each triol, as reflected by its intensity was unexpected in view of the relatively low intensities of the molecular ions of methandrostenolone and its 17-epimer. This difference can, however, be explained by the lack of any system to promote ring fragmentation in the saturated androstanetriols in contrast to the intense B-ring fragmentation caused by an A-ring dienone system.

The mass spectrum of a sample of 17α-methyl-5α-androstane-2α,3β,17-triol [figure 13b] differed from those of the other three triols. Maximum ion pressure was obtained from the sample at 105-140° in contrast to 170-180° for the latter compounds and the intensities of all ions in the high mass region were less than 10% of a very intense peak at m/e 347. The molecular ion of the triol (m/e 322, 3.5%) was present at 105° and, at 140°, the intensity of this ion relative to m/e 347 had risen to 11.5%; the ions at m/e 307

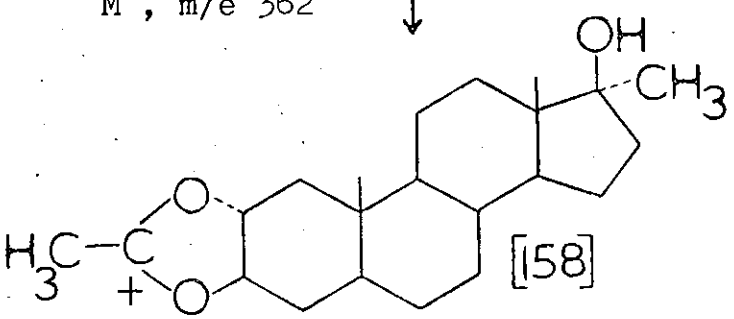


[146]



[157]

M^+, m/e 362

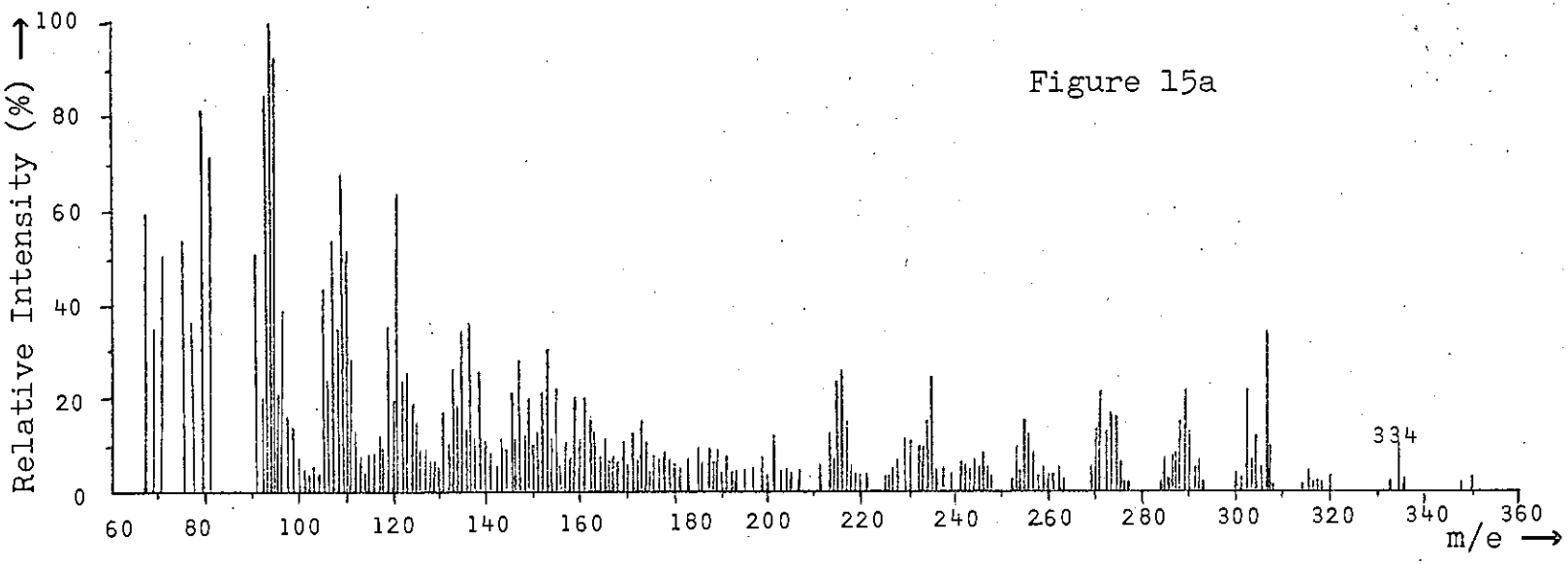


[158]

m/e 347

and m/e 304, characteristic of the other three triols were also detectable at this temperature. The spectra of the 2α,3β,17-triol were rationalised when a very low intensity ion (ca. 1% of m/e 347 at 105°) at m/e 362 was taken into account. This showed that spontaneous formation of the acetonide [157] of the diequatorial glycol system of 17α-methyl-5α-androstane-2α,3β,17-triol[146] had occurred during crystallisation from acetone. Loss of a methyl group from the acetonide (M^+, m/e 362) thus gave rise to the very intense ion at m/e 347 [158]. Exact mass measurement of the ions at m/e 362 and m/e 347 gave values in error of less than 0.1 p.p.m. and 1 p.p.m. for the $C_{23}H_{38}O_3$ and $C_{22}H_{35}O_3$ structures, respectively.

A sample of the 2α,3β-glycol which had been re-crystallised from acetone was dissolved in ethanol and water, heated and benzene was added. The solvents were removed by evaporation in vacuo, benzene was added and the evaporation repeated. The resulting material was examined by mass spectrometry at 140° and four spectra were recorded at intervals of one minute between each. The relative intensities of the M-15 ion of the acetonide (m/e 347) and M^+ of the triol (m/e 322) reversed during the time in which the spectra were recorded [see table 12], showing that the greater volatility of the acetonide was responsible for the lack of evidence of the triol in the spectrum obtained at 105°. Thus, as confirmed by t.l.c. and n.m.r. spectroscopy, the original sample of the triol contained only a small amount of the volatile acetonide derivative which masked the presence of the free triol.



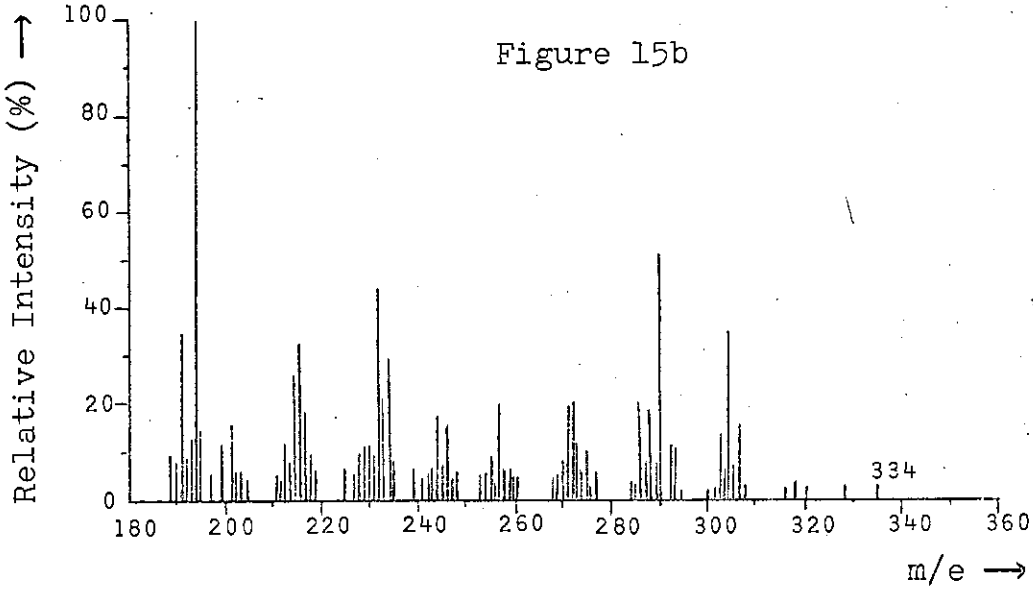


Figure 15. Mass spectra of samples of the less polar metabolite of oxymetholone.

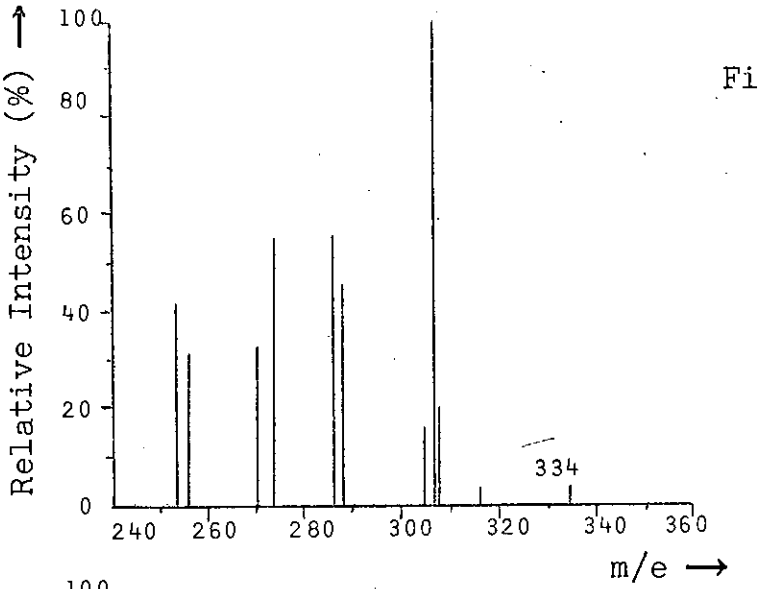


Figure 15c

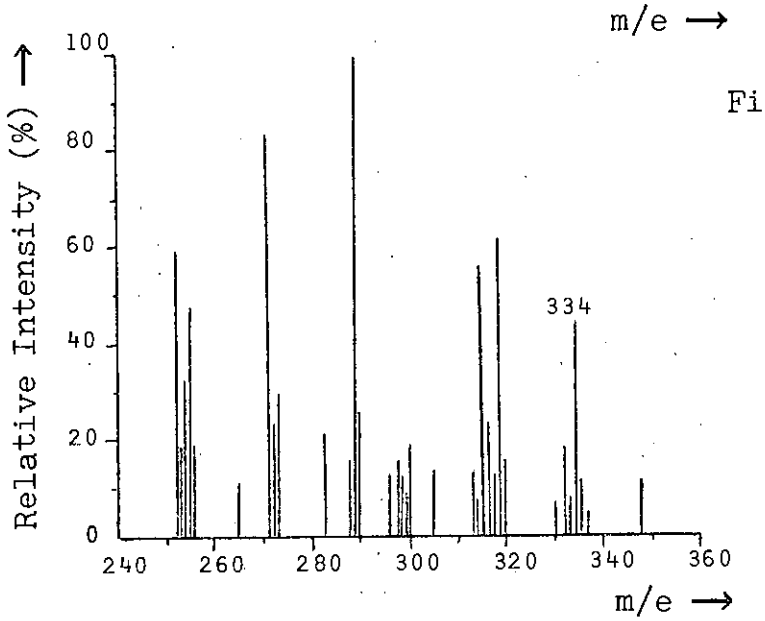


Figure 15d

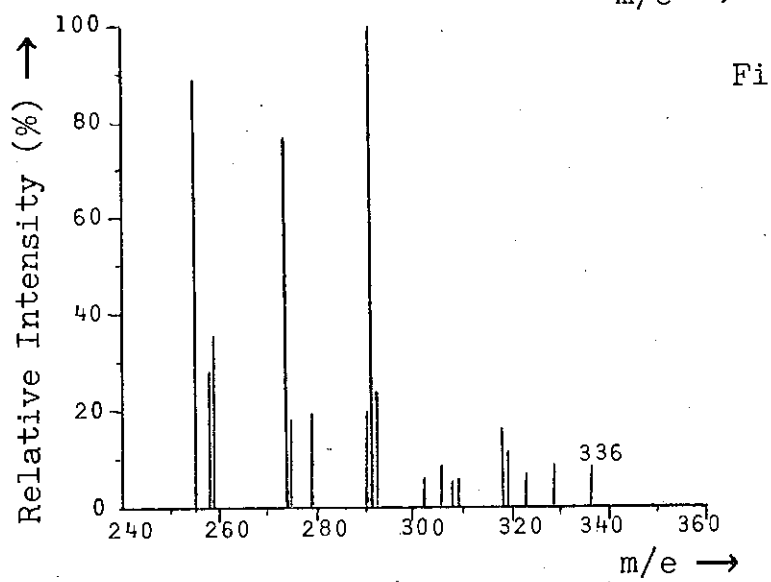


Figure 15e

Figure 15. Mass spectra of samples of the less polar metabolite of oxymetholone (c and d) and of the more polar metabolite (e).

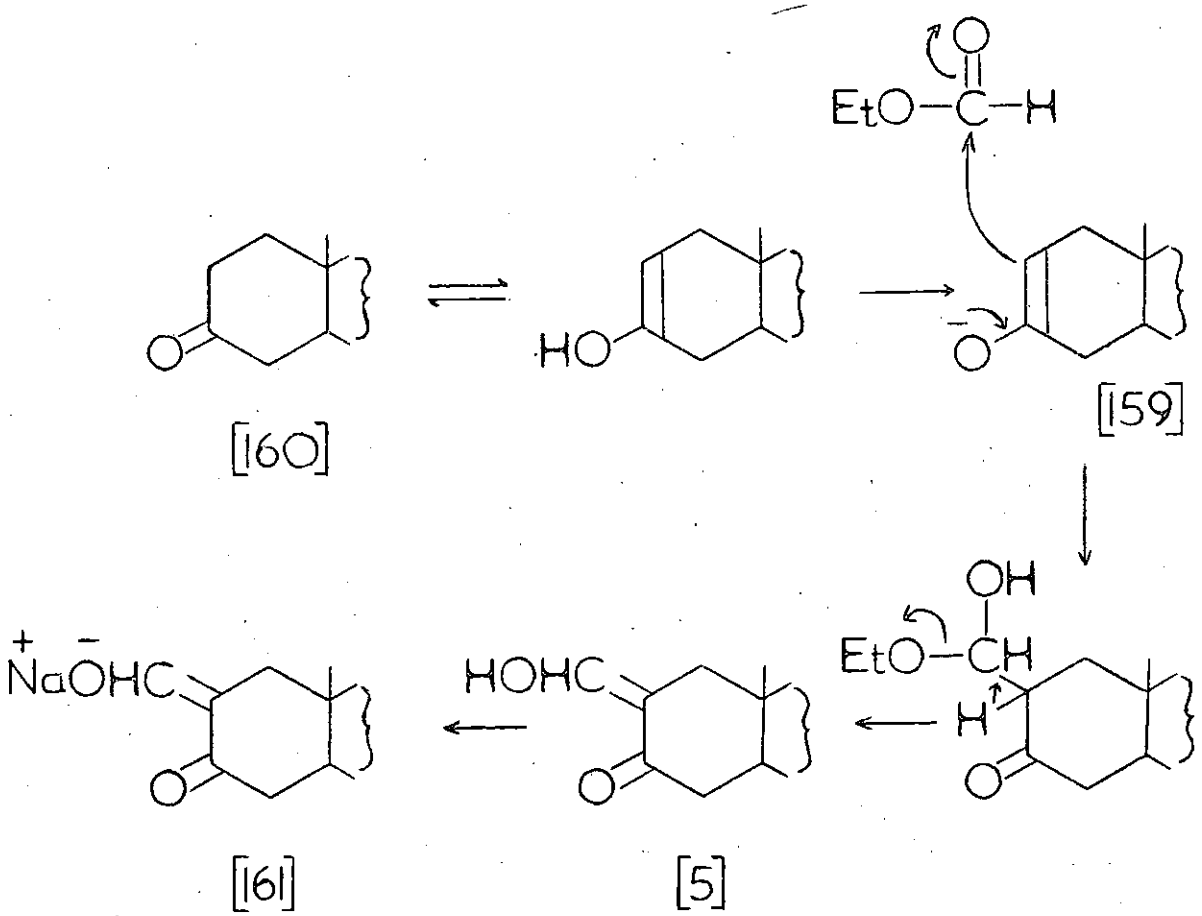
Spectrum	Time (mins.)	m/e 347	m/e 322	$\frac{m/e\,322}{m/e\,347}$
1	0	21	4	0.19
2	1	10	5	0.50
3	2	5.5	6.5	1.18
4	3	2	7.5	3.75

Table 12. Variations in the intensities of the ions at m/e 347 and m/e 322 in mass spectra of 17α-methyl-5α-androstane-2α,3β,17-triol with time.

Metabolic samples were obtained by thin layer and paper chromatography of urine extracts following the administration of 10mg doses of oxymetholone. Carbon skeleton chromatography was used to locate those fractions containing material with a 17α-methylandrostane skeleton and each metabolic sample was thought to contain only one of the two previously located[32] metabolites. Five such samples were examined by mass spectrometry and all gave spectra indicative of impure mixtures [figures 15a, 15b, 15c, 15d, 15e]. Many intense ions were present in each spectrum up to ca. m/e 306 but beyond this mass value those ions which might characterise metabolites of oxymetholone were of low intensity. However, the only region of the spectrum which could be used for characterisation of the metabolites was that above m/e 306 because the metabolites of oxymetholone, being presumably fully ring-saturated like the drug, were not expected to produce any characteristic medium mass ions of high intensity, such as the m/e 122, m/e 121 pair given by methandrostenolone and its metabolites.

None of the samples of metabolites gave ions at m/e 322 which might have indicated a 2,3,17-triol, nor were the characteristic m/e 307 and m/e 304 ions [see figures 13a, 13b, 13c, 13d] present. Consequently it was concluded that a 17α-methyl-5α-androstane-2,3,17-triol was not a metabolite of oxymetholone and that a 2,17β-dihydroxy-17-methyl-5α-androstan-3-one was also unlikely to be formed during metabolism.

Four of the spectra of the metabolites showed ions at m/e 334 [figures 15a, 15b, 15c, 15d]. In view of the reported microbial reductions[83,84] of a series of 2-hydroxymethylene-Δ^4-en-3-ones in the androstane series to the 2-hydroxymethyl-Δ^4-ene-3-ketones the observation of ions at m/e 334 in the spectra of metabolites of oxymetholone suggested that the 2-hydroxymethylene function might be metabolised to give a 17β-hydroxy-2-hydroxymethyl-17-methyl-5α-androstan-3-one (M^+, m/e 334). A spectrum [figure 15e] of a sample of the most polar metabolite of oxymetholone contained an ion at m/e 336 which could be assigned to a 2-hydroxymethyl-17-methyl-5α-androstane-3,17-diol (M^+, m/e 336), formed by reduction of the C-3 carbonyl function of oxymetholone as well as the 2-hydroxymethylene system. It would thus appear that during metabolism of oxymetholone the additional carbon atom at C-2 is retained. The reductions of oxymetholone with a variety of reagents, to provide standard compounds, are therefore described in the following section.



[160] [159] [5] [161]

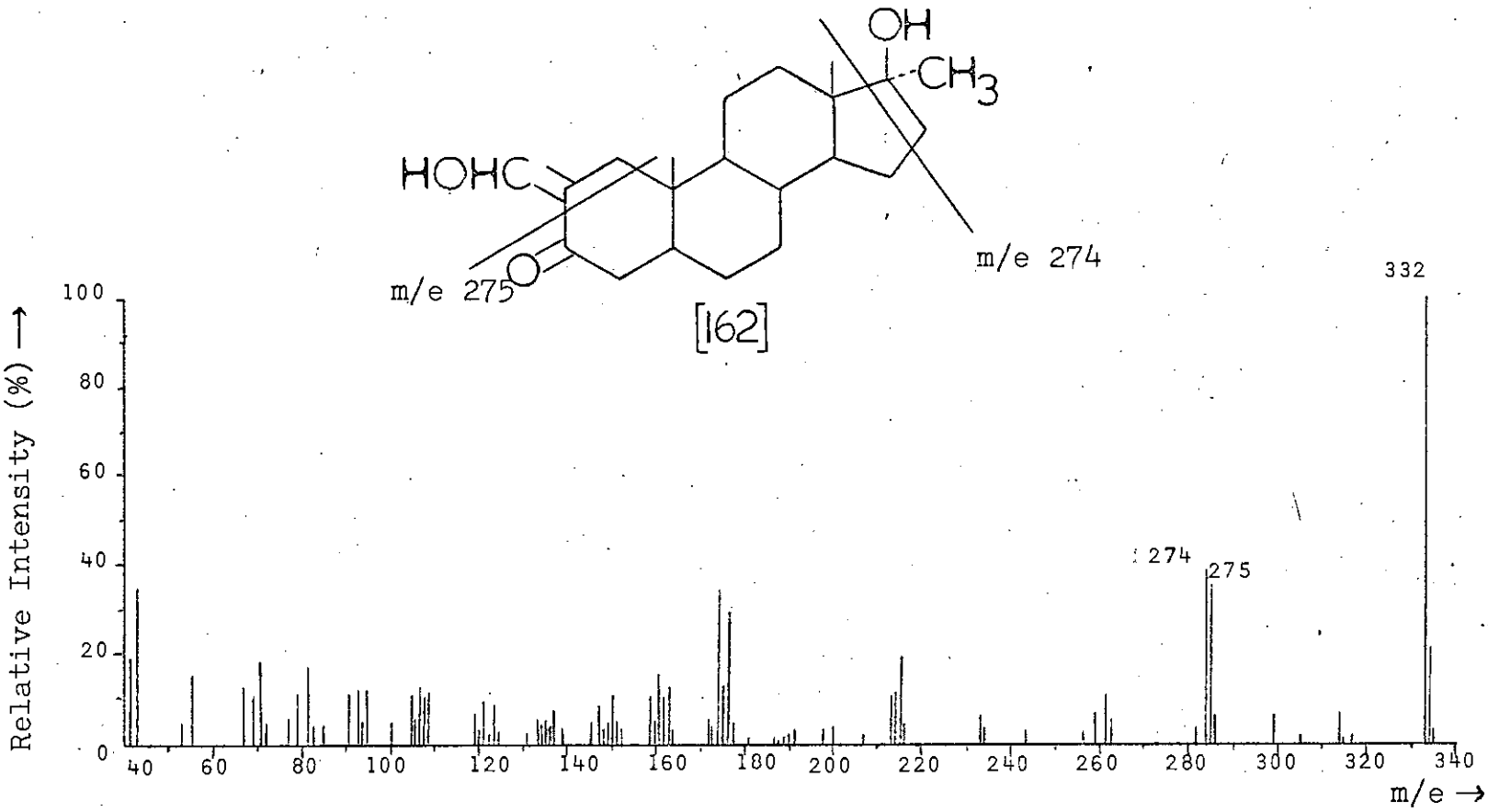


Figure 16. The mass spectrum of oxymetholone at 120°

4.4. The synthesis and chemical reductions of oxymetholone

4.4.i. Synthesis of oxymetholone

Oxymetholone was synthesised to provide a more readily available source of the compound in greater quantity than that obtained by extraction of commercial tablets of the drug. The preparation of oxymetholone[5] was first described by Ringold *et al.*[20] and is achieved by condensation of ethyl formate with the Δ^2-enolate anion [159] of 17β-hydroxy-17-methyl-5α-androstan-3-one [160]. The product is precipitated as the enolate salt [161] from the non-polar solvent and then acidified to give[117m] the free hydroxymethylene compound [5]. 17β-Hydroxy-17-methyl-5α-androstan-3-one[160] was prepared from 3β-hydroxy-5α-androstan-17-one by reaction with methylmagnesium iodide followed by chromic acid oxidation of the C-3 hydroxyl group of 17α-methyl-5α-androstane-3β,17-diol.

In the mass spectrum of oxymetholone [figure 16] the molecular ion (M^+, m/e 332) was also the base peak and no other ions were greater than 40% of M^+. The intensities of M-18 (m/e 314, 7%) and M-18-15 (m/e 299, 7%) reflect the low tendency of the 17β-hydroxy-17-methyl function to lose water, whereas the medium intensity ion at m/e 274 (M-58, 38%) shows that D-ring fragmentation is more favourable for this configuration (*cf.* m/e 242 in the spectrum of methandrostenolone [figure 5a]). The ion at m/e 275 (36%) presumably arises from fission of the 2,3-bond, hydrogen transfer and loss of the hydroxymethylene function with the C-2 and C-1 carbon atoms [162].

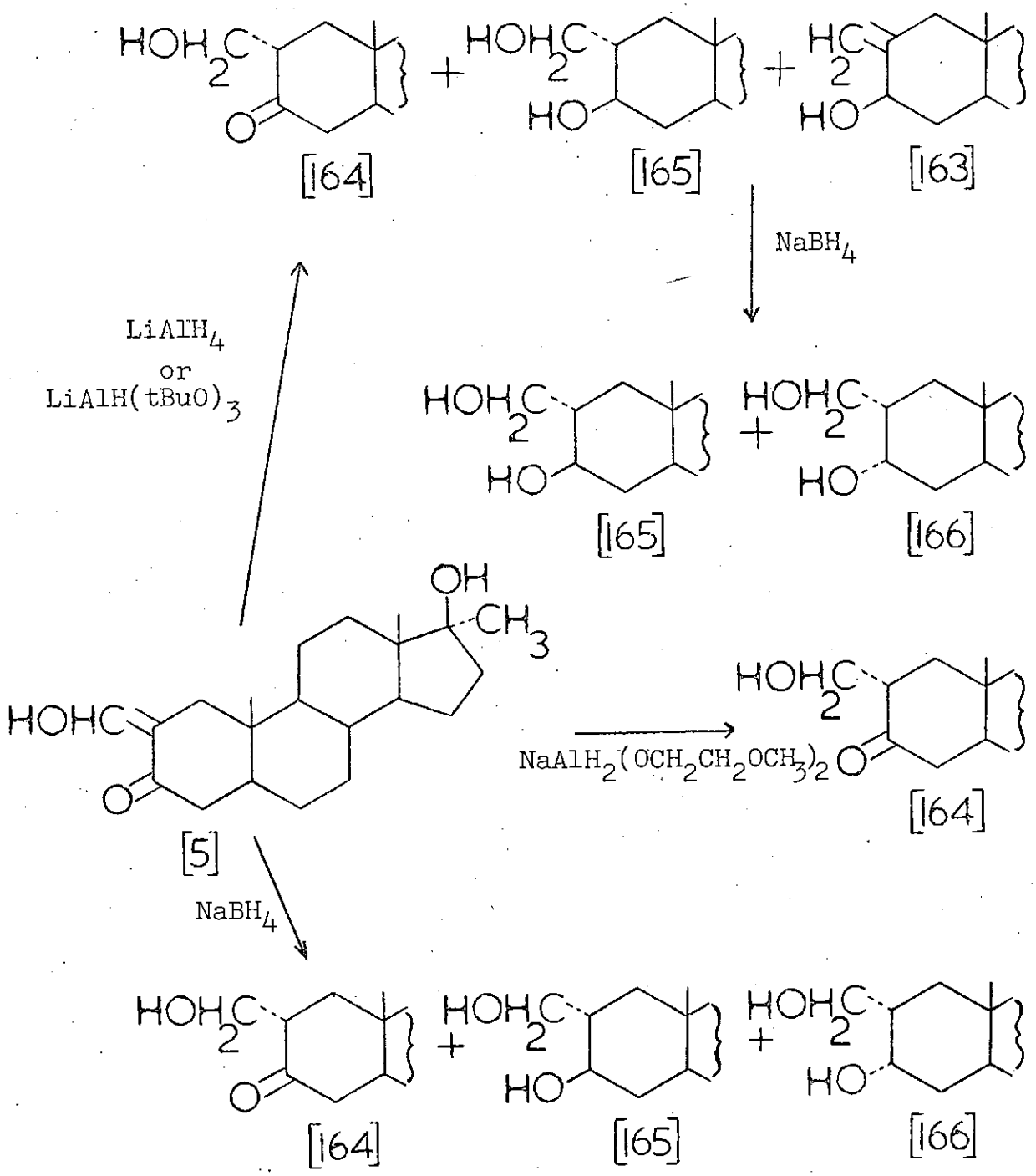


Diagram 19. The products of the reduction of oxymetholone with metal hydrides.

4.4.ii. Reduction of oxymetholone with lithium aluminium hydride

Oxymetholone was reduced with lithium aluminium hydride at room temperature as described by Knox and Velarde.[206] When the product was isolated by the addition of sodium sulphate and removal of the salt cake by filtration as described[206] by these authors only 87% of the steroid was recovered. Consequently, the salt cake was dissolved in water and extracted with ether by which means the remaining product, rich in the most polar, least ether-soluble component was isolated.

T.l.c. showed that both product fractions contained the same four compounds, the least polar of which was unreacted oxymetholone[5]. The other three components were identified by comparison with the products obtained by Knox and Velarde.[206] In order of increasing polarity these were 17α-methyl-2-methylene-5α-androstane-3β,17-diol[163], 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one[164] and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol[165]. Crystallisation of the major fraction from acetone gave 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one[164], m.p. 183-185° (lit.,[206] 188-190°) which was shown by t.l.c. to be slightly contaminated with 17α-methyl-2-methylene-5α-androstane-3β,17-diol[165]. Pure 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol[165], m.p. 280-282° (lit.,[206] 280-282°) was obtained by crystallisation of the minor fraction from acetone.

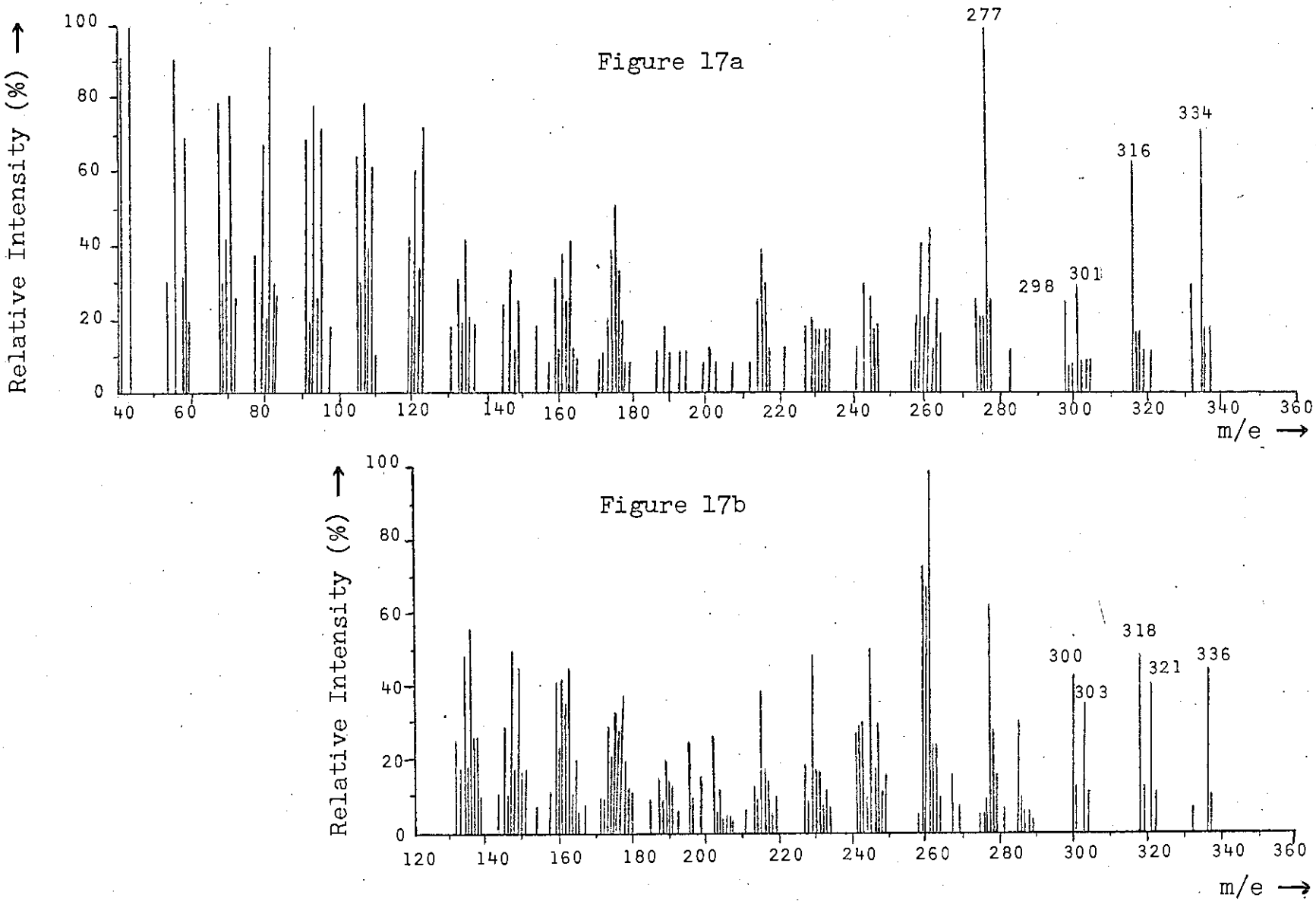


Figure 17. The mass spectra of a) 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and b) 2α-hydroxymethyl-17-methyl-5α-androstane-3β,17-diol both at 160°C.

4.4.iii. Reduction of oxymetholone with lithium tri-t-butyl-oxyaluminium hydride

Oxymetholone[5] was reduced with lithium tri-t-butyl-oxyaluminium hydride to give a crude product from which a mixture of 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one[164] and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β, 17-diol[165] was crystallised as the major product. The crude product contained some unreacted oxymetholone and a small amount of the 2-methylene-3β-ol[163].

The 2α-hydroxymethyl-3-ketone[164] was more volatile than the 2α-hydroxymethyl-3β-ol[165], mass spectrometry of the mixture giving spectra with M^+ at m/e 334 [Figure 17a, 160°] and at m/e 336 (180°). The base peak in the spectrum [figure 17a] of the former compound was at m/e 277, formed by loss of a fragment of mass 57 from the molecular ion. One of the major peaks in the spectrum of oxymetholone [figure 16] was likewise formed by loss of a mass 57 fragment. The other important ions in the spectrum of the 2α-hydroxymethyl-3-ketone were formed by loss of water from the molecular ion (m/e 316) and of water (m/e 298) or a methyl group (m/e 301) from this ion.

Acetylation of the mixture of the 2α-hydroxymethyl-3-ketone and the 2α-hydroxymethyl-3β-ol with acetic anhydride in pyridine at room temperature gave a mixture containing two low-polarity components, the mass spectrum of which contained the molecular ions of the monoacetate (m/e 376) and the diacetate (m/e 420). Under these conditions the tertiary 17β-hydroxyl function was not acetylated.

The mixture containing the 2α-hydroxymethyl-3-ketone and the 2α-hydroxymethyl-3β-ol was reduced with sodium borohydride. T.l.c. of the product showed that the less polar 3-ketone [164] had been reduced to the 2α-hydroxymethyl-3β-ol[165] and a trace of a slightly more polar compound which was presumed to be the epimeric 2α-hydroxymethyl-3α-ol[166]. This was confirmed by mass spectrometry of the product which showed only a molecular ion at m/e 336 in spectra obtained at a variety of temperatures. [see figure 17b]. Other characteristic ions in the spectra of the 2α-hydroxymethyl-3-ol were those arising from loss of water or a methyl group from the molecular ion (m/e 318 and m/e 321, respectively) and from loss of water from both of these ions (m/e 300 and m/e 303).

The product of the sodium borohydride reduction was acetylated to give a product which showed one low-polarity spot on t.l.c. and gave a mass spectrum with the molecular ion of a 2-acetoxymethyl-3-acetate (m/e 420). The spectrum contained no trace of the molecular ion of the monoacetate at m/e 376.

4.4.iv. Reduction of oxymetholone with sodium borohydride

Oxymetholone [5] was reduced with sodium borohydride to give a crude product, the components of which were identified by t.l.c. against standards from the preceding reductions. The product contained unreacted oxymetholone [5], 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one[164] and a mixture of the two epimeric 2α-hydroxymethyl-17α-methyl-5α-androstane-3,17-diols [165,166].

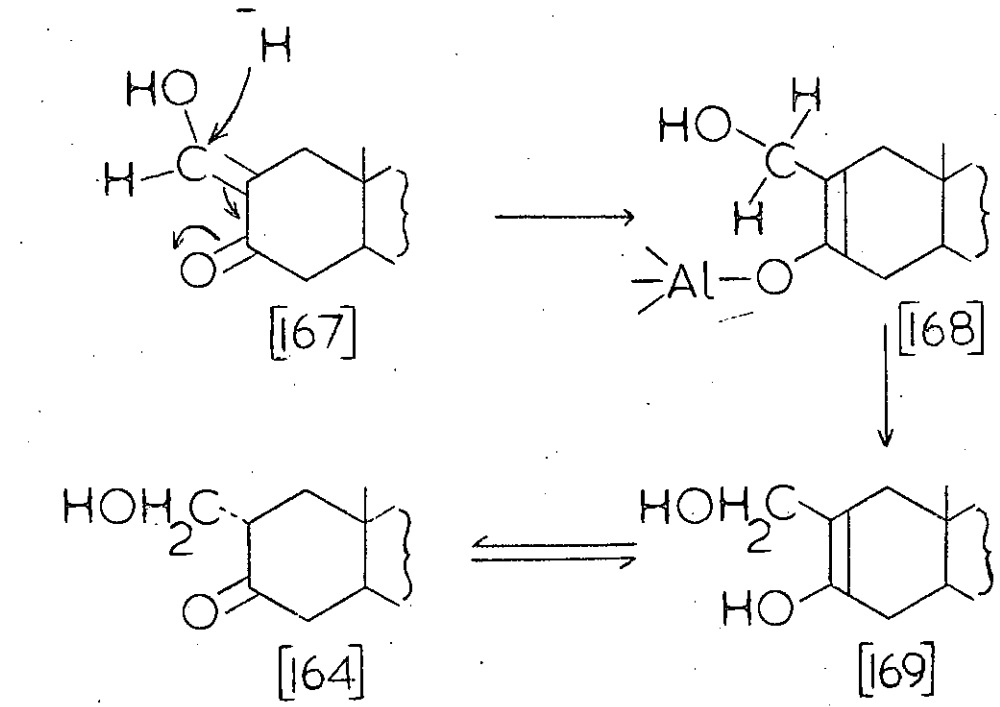
4.4.v. Reduction of oxymetholone with sodium dihydro-bis (2-methoxyethoxy)aluminate

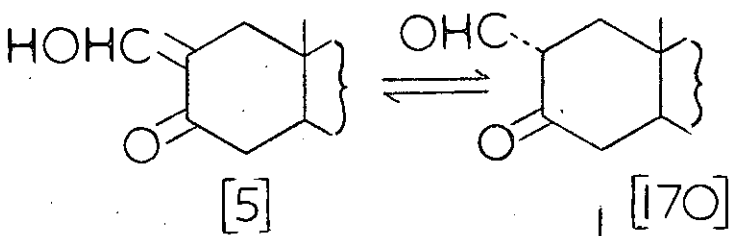
Reduction of oxymetholone with sufficient sodium dihydro-bis(2-methoxyethoxy)aluminate for reaction of the 2-hydroxymethylene and 3-carbonyl functions gave a crude product containing no unreacted oxymetholone. However, the major product was identified by t.l.c. as 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one[164] accompanied by only traces of more polar components. Use of sufficient reagent for complete reduction of only one of the functions gave a mixture containing unreacted oxymetholone, traces of polar components and the 2α-hydroxymethyl-3-ketone[164] as the major product.

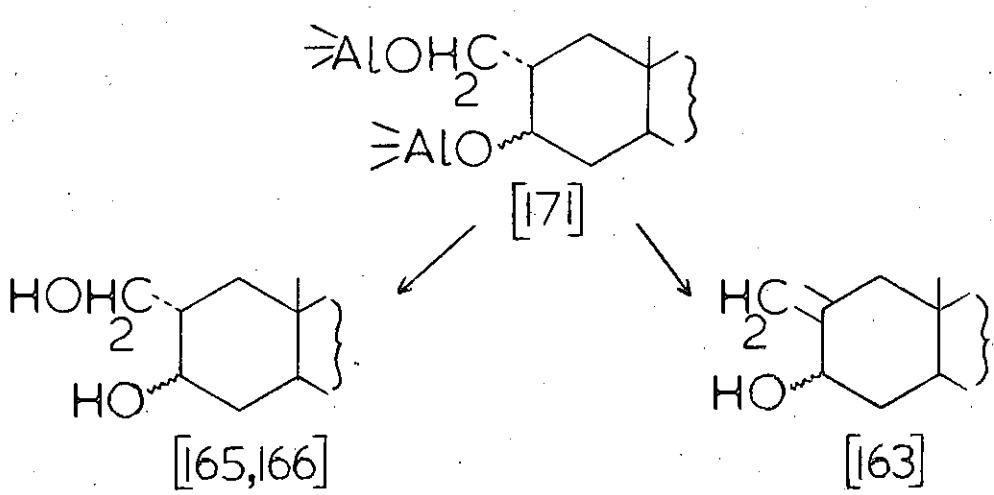
4.4.vi. Discussion

The reductions of oxymetholone with lithium aluminium hydride, lithium tri-t-butyloxyaluminium hydride, sodium borohydride and sodium dihydro-bis(2-methoxyethoxy)aluminate are of interest because of the different product mixtures obtained and the nature of the products.

The major product of the reduction with all four reagents was 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one. Isolation of a ketonic product from a reduction in which excess reducing agent was employed must be ascribed to the relationship of the two reducable groups in the oxymetholone molecule. Thus, conjugation of the hydroxymethylene double bond with the 3-carbonyl function leads to a positive centre at C-2' because of polarisation towards the carbonyl oxygen atom. This centre is then attacked by


H
HO
H
O
[167]
HO
H
H
Al–O
[168]
HOH2C
O
[164]
HOH2C
HO
[169]


HOHC
O
[5]
OHC
O
[170]


AlOH2C
AlO
[171]
HOH2C
HO
[165,166]
H2C
HO
[163]

a hydride ion [167] with the generation of a Δ^2-double bond and formation of an O-Al bond at C-3 [168]. Consequently, further attack by H^- cannot occur and the addition of water leads to hydrolysis of the O-Al bond with formation of an enol [169] which then isomerises to give the 2α-hydroxymethyl-3-keto compound [164].

The formation of 3-hydroxy-2α-hydroxymethyl compounds may be explained by hydride reduction of the 2-aldehyde function [170] present in equilibrium with the 2-hydroxymethylene system [5] in a proportion (ca. 22%, by calculation from n.m.r. spectra[204b]) of the sample, followed by reduction of the 3-carbonyl group. Alternatively the latter function may be attacked by a hydride ion with destruction of the hydrogen bonding with the 2-hydroxymethylene system and consequent isomerisation to the aldehyde which is then reduced. The 2-methylene product [163] is formed by elimination of the 2'-OAl substituent from the intermediate to the 2α-hydroxymethyl-3-alcohol[171].

Sodium dihydro-bis(2-methoxyethoxy)aluminate reacted with oxymetholone to give only the 2α-hydroxymethyl-3-one. Consequently the low yields of the 2α-hydroxymethyl-17α-methyl-5α-androstane-3,17-diols in the reactions with the other three reagents must be due to a combination of the faster hydride attack at C-2', because of the conjugated enone system, than at C-3 and to the steric requirements for the formation of the two O-Al bonds.

The reduction of oxymetholone to a 2α-hydroxymethyl-3β-alcohol with lithium aluminium hydride and with lithium

tri-t-butyloxyaluminium hydride and of both oxymetholone and 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one to mixtures of the 3α- and 3β- alcohols with sodium borohydride is not surprising. The anion from lithium aluminium hydride in ether is much less heavily solvated than that from sodium borohydride in a hydroxylic solvent[117a] so that Dauben's "steric approach factor"[207] is smaller for the former reagent than the latter. Consequently more of the thermodynamically less stable alcohol is formed by reaction with sodium borohydride. Eliel and Rerick[208] observed that sterically unhindered 3-ketones and analogues such as 4-t-butyl cyclohexanone gave a greater proportion of the equatorial alcohols by reduction with lithium aluminium hydride in ether than with sodium borohydride in hydroxylic solvents.

Dreiding and Hartman[209] studied the lithium aluminium hydride reduction of 2-carbethoxycyclohexanone and 2-hydroxymethylenecyclohexanone and suggested a mechanism whereby the enolised portions of the compounds reacted to give enolate salts whereas the non-enolic portions were reduced normally to give diols. They isolated 2-methylenecyclohexanol, 1-cyclohexenemethanol and 2-hydroxymethylcyclohexanol in the ratio 5:2:1 from the reductions of the above compounds. However no 2-hydroxymethyl-Δ^2-ene has been found among the reduction products of oxymetholone.[206]

4.4.vii. Comparison of the metabolites of oxymetholone with 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and the 2α-hydroxymethyl-17α-methyl-5α-androstane-3,17-diols.

The mass spectra of four samples of the less polar

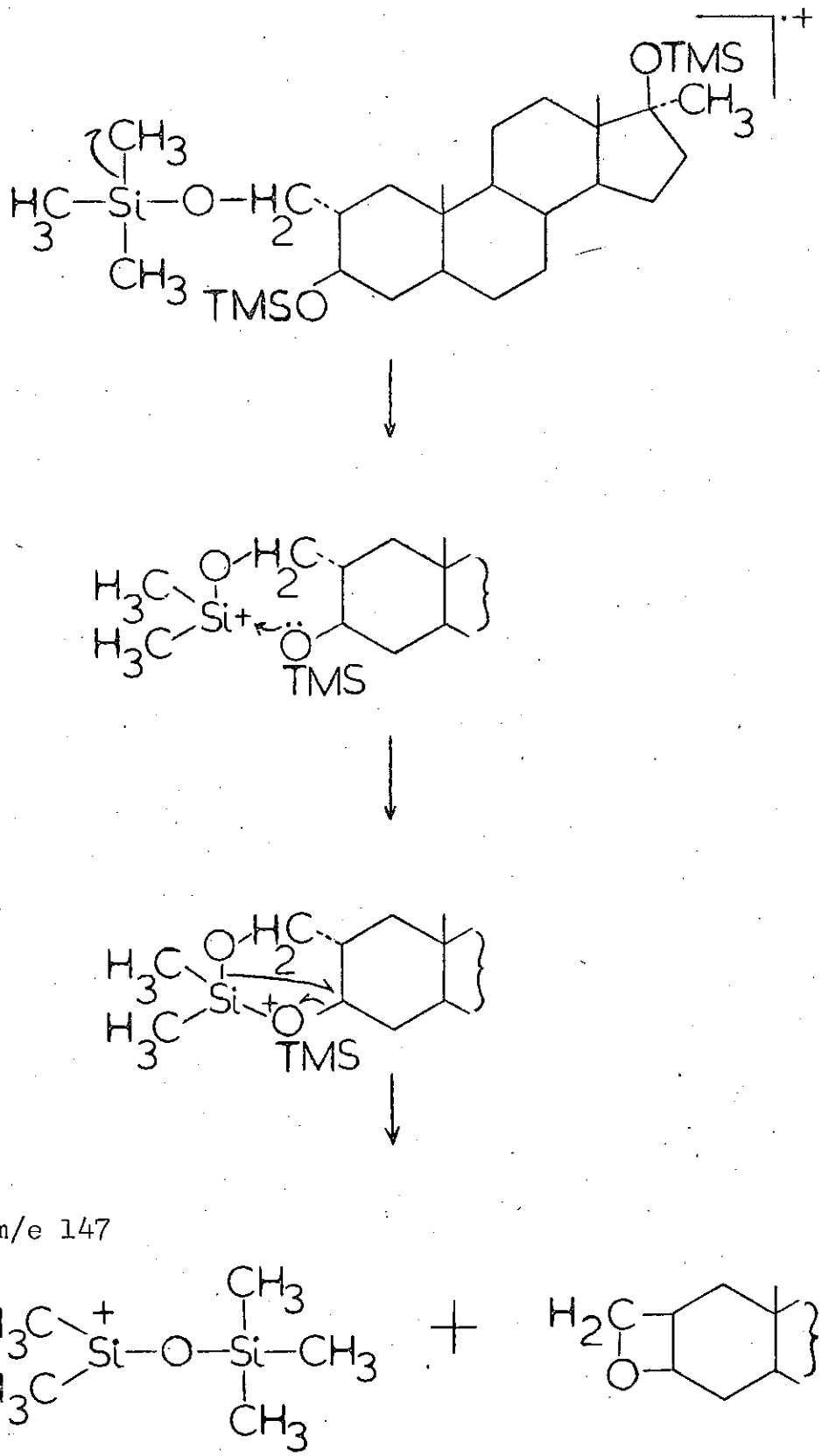


Diagram 20. The mechanism of the formation of the m/e 147 fragment in the mass spectra of compounds containing two adjacent trimethylsilyl ether fractions.

metabolite of oxymetholone [figures 15a, 15b, 15c, 15d] contained ions at m/e 334 (M^+ of 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one, [figure 17a]) and fairly prominent ions at m/e 316 (M-18). The sample of the most polar metabolite [figure 15e] gave an ion at m/e 336 (M^+ of 2α-hydroxymethyl-17α-methyl-5α-androstane-3,17-diol, [figure 17b]) and a pronounced ion at m/e 318 (M-18). Spectra 15d and 15e corresponded to the two metabolite-containing fractions obtained after a single administration of oxymetholone. Both fractions were chromatographed on a thin layer plate against 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one (R_f 0.53), 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol (R_f 0.38) and 2α-hydroxymethyl-17α-methyl-5α-androstane-3α,17-diol (R_f 0.31) as standards. The sample containing the most polar metabolite gave a spot at R_f 0.38 and that containing the least polar metabolite gave a spot at R_f 0.50 with others at 0.44 and 0.24.

Mass spectrometry of the trimethylsilyl derivatives of the samples from which spectra 15d and 15e were obtained tended to confirm the proposed structures of the metabolites. The sample containing the more polar metabolite gave a relatively intense ion at m/e 147, not present in the spectrum of the free steroid. Such an ion is formed[210] by the interaction of the trimethylsilyl functions of two adjacent hydroxyl groups through a cyclic intermediate with expulsion of the $SiMe_3O\overset{+}{S}iMe_2$ fragment (m/e 147) [see diagram 20]. An ion at m/e 147 was not observed in the spectrum of the derivative of the less polar metabolite. An intense

ion at m/e 143, characteristic of the 17β-hydroxy-17-methyl system was not found in the spectrum of either derivative, possibly due to more intense fragmentations, such as that giving m/e 147, induced by the silyl groups in the A-ring.

Carbon skeleton chromatography of a sample of 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol, obtained by lithium aluminium hydride reduction of oxymetholone (section 4.5.iv), gave only reduced products with the retention times of 17-methylandrostanes. Thus, the 2α-hydroxymethyl-, like the 2-hydroxymethylene-function is removed during high temperature catalytic reduction and consequently all available evidence indicates that the two metabolites of oxymetholone in normal man are 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol, although the analysis of further samples is desirable.

4.5. Experimental Section

4.5.i. 17α-Methyl-5α-androstane-3β,17-diol

A solution of methyl iodide (52g, 23ml) in dry ether (150ml) was added to magnesium turnings (9g) in ether (50ml) and the mixture refluxed for 15 minutes. 3β-Hydroxy-5α-androstan-17-one (10g, for preparation, see section 4.2.i.) was dissolved in ether (500ml) and a small volume of tetrahydrofuran and added dropwise to the stirred solution of methylmagnesium iodide. After refluxing for 15 minutes the reaction mixture was poured into ice, water and concentrated sulphuric acid (40ml) and stirred until all the ice had melted. The layers were separated and the aqueous layer was extracted three times with chloroform-ether (1:9). The combined organic extracts were washed with sodium sulphite solution, saturated sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give the product (9.665g), a mixture of the starting material and the diol. Pure 17α-methyl-5α-androstane-3β,17-diol (7.711g, 73%) was obtained by recrystallisation from acetone; m.p. 211.5-212.5° (lit.,[211] 206-209°); n.m.r. (60 M Hz) τ9.18 (C-19 methyl), 9.16 (C-18 methyl), 8.80 (C-20 methyl), 6.45 (m, 3α-H).

4.5.ii. 17β-Hydroxy-17-methyl-5α-androstan-3-one

8N Chromic acid solution (5ml) was added to a stirred solution of 17α-methyl-5α-androstane-3β,17-diol (2.502g) in acetone (500ml). The reaction was allowed to proceed for 1.5 minutes and then quenched with methanol (500ml). Water (500ml) was added and the mixture neutralised with saturated

sodium bicarbonate solution. The organic solvents were removed by evaporation *in vacuo*, the aqueous phase was extracted twice with ether and the combined organic extracts were washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a yellow solid. Crystallisation from acetone gave pure 17β-hydroxy-17-methyl-5α-androstan-3-one (1.674g) as needles; m.p. 192-194° (lit.,[111b] 193°); ν_{max} 1710cm^{-1}; n.m.r. (60 M Hz) τ9.14 (C-18 *methyl*), 8.98 (C-19 *methyl*), 8.81 (C-20 *methyl*).

4.5.iii. *Oxymetholone*[5][20]

Sodium hydride (2g, 60% dispersion in oil) was washed free from oil with petrol (60-80°) and then with benzene and added to a stirred mixture of 17β-hydroxy-17-methyl-5α-androstan-3-one (1.009g), dry benzene (20ml) and ethyl formate (2ml) under nitrogen. After 5 hours the green reaction mixture was filtered, the precipitate was washed with benzene and with hexane, dried and added to ice-cold dilute hydrochloric acid (50ml). The lumps were crushed and the mixture was again filtered. The precipitate was washed with water, dissolved in ether and the solution washed twice with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a yellow glass. Crystallisation from acetone gave pure oxymetholone (0.660g); m.p. 174-178° (lit.,[20] 178-180°); ν_{max} 3380,2920,1640,945cm^{-1}; n.m.r. (100 M Hz) τ9.23 (C-19 *methyl*), 9.14 (C-18 *methyl*), 8.80 (C-20 *methyl*), 1.41 (s, average of $-\overset{\backsim}{C}O.\overset{\backsim}{C} = C.\underline{H}.OH$ and $-\overset{\backsim}{C}.OH = \overset{\backsim}{C}.\underline{C}HO$[204b]); Mass spectrum, M^+ and base peak, m/e 332.

4.5.iv. Reduction of oxymetholone with lithium aluminium hydride[206]

A fine suspension of oxymetholone (501.6mg) in ether (50ml) was added dropwise to a stirred suspension of lithium aluminium hydride (497.1mg) in ether (150ml) and the mixture was stirred at room temperature for 1½ hours. Excess hydride was destroyed with ethyl acetate and then saturated aqueous sodium sulphate (1ml) was added, followed by sufficient sodium sulphate to give a clear supernatant ether solution. The mixture was filtered, the salt cake was washed with ether and the combined filtrate and washings were evaporated to dryness to give a product (436.6mg, 87%). The salt cake was dissolved in water, the solution extracted twice with ether and the combined organic extracts washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a sparingly soluble white material (81.7mg, 16%). T.l.c. of both products showed the same four major components, in order of increasing polarity; oxymetholone, 17α-methyl-2-methylene-5α-androstane-3β,17-diol, 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol. The smaller fraction (81.7mg) extracted from the salt cake appeared to be richer in the latter compound and crystallisation from acetone gave pure 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol; m.p. 280-282° (lit.,[206] 280-282°). The major component of the other fraction was the 2α-hydroxymethyl-3-ketone and crystallisation from acetone gave 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one; m.p. 183-185° (lit.,[206] 188-190°); t.l.c., slightly contaminated with 17α-

methyl-2-methylene-5α-androstane-3β,17-diol; ν_{max} 1700cm^{-1}; n.m.r. (100 M Hz) τ9.16 (C-19 methyl), 9.14 (C-18 methyl), 8.81 (C-20 methyl), 6.34 (m, 2β-H), 6.14 (m, -CH_2OH); Mass spectrum, M^+, m/e 334.

4.5.v. Reduction of oxymetholone with lithium tri-t-butyl-oxyaluminium hydride[212]

Dry t-butanol (1.32ml) was added dropwise to a solution of lithium aluminium hydride (179.4mg) in tetrahydrofuran (5.9ml). This solution was then added dropwise to a solution of oxymetholone (592.2mg) in tetrahydrofuran (5.9ml) at 0°C and the reaction maintained at this temperature for 15 minutes. Excess reagent was decomposed with 5% acetic acid (70ml) and the steroid was extracted into ether, washed with sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give the crude product (571.5mg). Crystallisation from acetone gave a mixture of 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one (R_f 0.34) and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol (R_f 0.21, compared with oxymetholone, R_f 0.45, in benzene-ethanol, 9:1); Mass spectrum, M^+, m/e 334 (160°); M^+, m/e 336 (180°).

4.5.vi. Acetylation of 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol.

The product of the above reaction (18mg) containing a mixture of the 2α-hydroxymethyl-3-ketone and 2α-hydroxymethyl-3β-ol was dissolved in pyridine (0.05ml) and acetic anhydride (0.1ml). After 12 hours at room temperature

ether and water were added, the ether layer was washed with saturated sodium bicarbonate solution, dried over anhydrous magnesium sulphate and evaporated to dryness to yield 2α-acetoxymethyl-17β-hydroxy-17-methyl-5α-androstan-3-one and 2α-acetoxymethyl-17α-methyl-5α-androstane-3β,17-diol 3-acetate (17.2mg); t.l.c., two spots, both less polar than the starting materials; ν_{max} 1740,1710cm^{-1}; Mass spectrum, M^+ of monoacetate, m/e 376; M^+ of diacetate, m/e 420.

4.5.vii. Reduction of 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one and 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol with sodium borohydride[118]

The product of reaction 4.5.v. (1.1mg) containing a mixture of the 2α-hydroxymethyl-3-ketone and 2α-hydroxymethyl-3β-ol was dissolved in methanol (0.05ml) and ethyl acetate (0.01ml) and mixed with a solution of sodium borohydride (0.3mg) in methanol (0.05ml) and ethyl acetate (0.01ml). After 12 hours at room temperature glacial acetic acid (3 drops) and dilute hydrochloric acid were added. The steroid was extracted into ether, washed with sodium bicarbonate solution and with water, dried over anhydrous magnesium sulphate and evaporated to dryness to yield a white crystalline material (1.0mg); m.p. 260°; t.l.c., no spot corresponding to the 2α-hydroxymethyl-3-one but a spot corresponding to the 2α-hydroxymethyl-3β-ol and a very faint, slightly more polar spot presumed to be 2α-hydroxymethyl-17α-methyl-5α-androstane-3α,17-diol; Mass spectrum, M^+, m/e 336.

4.5.viii. Acetylation of 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol

The product of the above reaction (1mg) was dissolved in pyridine (3 drops) and acetic anhydride (6 drops). After 7 hours the product was isolated as described in section 4.5.vi. to give 2α-acetoxymethyl-17α-methyl-5α-androstane-3β, 17-diol 3-acetate (1mg); t.l.c., one low polarity spot; Mass spectrum, M^+, m/e 420, with no trace of the mono-acetate (M^+, m/e 376).

4.5.ix. Reduction of oxymetholone with sodium borohydride[118]

A solution of sodium borohydride (73.1mg) in methanol (8ml) and ethyl acetate (2ml) was added to a solution of oxymetholone (260.7mg) in methanol (8ml) and ethyl acetate (2ml). After 24 hours at room temperature glacial acetic acid (4ml) and dilute hydrochloric acid were added and the product (266.2mg) isolated as described in section 4.5.vii. T.l.c. showed that it was a mixture of unreacted oxymetholone, 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one, 2α-hydroxymethyl-17α-methyl-5α-androstane-3β,17-diol and 2α-hydroxymethyl-17α-methyl-5α-androstane-3α,17-diol by comparison with the appropriate standards from the preceding reactions.

4.5.x. Reduction of oxymetholone with sodium dihydro-bis-(2-methoxyethoxy)aluminate

A 70% solution of sodium dihydro-bis(2-methoxyethoxy) aluminate in benzene (0.5ml, ca. 4 equivalents of H, Cambrian Chemicals Ltd., Croydon) was added dropwise to a stirred solution of oxymetholone (268.1mg) in benzene (25ml).

After 1 hour excess reagent was destroyed by the addition of water. Ether and sodium bicarbonate solution were added and the aqueous phase was extracted twice with ether. The combined organic extracts were washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a pale yellow glass (275.2mg). T.l.c. showed that no unreacted oxymetholone remained and that the major product was 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one, with traces of several more polar components.

5.1. The reactions of 5β-androstane-3,17-dione, oxymetholone and methandrostenolone with liver alcohol dehydrogenase

5.1.i. Chemical[206] and microbial[83,84] reductions of the 2-hydroxymethylene substituent of steroids in the androstane series have been shown to produce the 2α-configuration of the resulting hydroxymethyl function, the equatorial conformation of the large group being thermodynamically more stable than the axial. Consequently, by analogy, the hydroxymethyl function in any metabolite of oxymetholone may be assigned the 2α-configuration. In contrast, the configuration of the 3-hydroxyl group in a metabolite of molecular weight 336, in which both the hydroxymethylene and 3-carbonyl functions are reduced, cannot be as easily assigned although the evidence presented in section 4.4 suggested a 3β-hydroxyl group.

Studies with rats have shown that testosterone is metabolised[213] to androstanetriols containing the 3α-hydroxyl and the 3β-hydroxyl configurations and that an epimerase system which catalyses the transformation of 5α-androstane-3α,17β-diol to 5α-androstane-3β,17β-diol can be induced.[214,215] Consequently, although biochemical reduction of a 5α-3-ketosteroid often leads to formation of the corresponding 3α-alcohol the configuration of a 3-hydroxyl group in a metabolite of oxymetholone cannot be assigned by analogy, particularly since the effect of the 2-hydroxymethyl substituent is unknown. The biochemical reduction of oxymetholone was thus investigated with the hope that sufficient of one of the 2α-hydroxymethyl-17α-methyl-5α-androstane-3,17-diols would be formed to enable its characterisation and hence, by

analogy, the tentative identification of the more polar metabolite of oxymetholone in man.

Liver alcohol dehydrogenase (LADH) has been shown to be specific for the dehydrogenation of the 3β-hydroxyl group of 3-hydroxy-5β-cholanic acids and to accomplish the reverse reduction of the ketone to the 3β-alcohol.[216] Data was not available for the reduction of 5α-3-ketosteroids with this enzyme system and consequently the reaction of 5β-androstane-3,17-dione was examined prior to the attempted reduction of oxymetholone to establish whether the reaction conditions were suitable.

Ultraviolet spectroscopy is often used to follow the course of enzymatic reductions by the disappearance of the 340 nm band of β-dihydro nicotinamide adenine dinucleotide (NADH) due to simultaneous oxidation of this cofactor.[216] However this method requires an excess of steroid so that the change in reduced cofactor concentration is significant, whereas the attempted reductions described in this section were best performed with a large excess of NADH. Consequently, reduction was monitored by t.l.c. and g.l.c. of the products extracted after incubation with the enzyme.

5.1.ii. The reaction of 5β-androstane-3,17-dione with LADH

5β-Androstane-3,17-dione was incubated with LADH and NADH in 2-amino-2-methylpropan-1-ol buffer (AMP, pH 10.5) at 30° for 12 hours. A control reaction, containing all the ingredients except the steroid was treated likewise.

Prior to termination of the incubation a small sample of the steroid reaction mixture was withdrawn, diluted with

buffer and incubated for 30 minutes with acetaldehyde. The ultraviolet spectrum of this solution did not contain a band at 340 nm whereas the spectrum of the reaction mixture which was not incubated with acetaldehyde possessed this band. Thus the enzyme system had remained active throughout the 12 hour incubation with steroid and was able to catalyse the reduction of acetaldehyde to ethanol with simultaneous oxidation of NADH to NAD. The enzyme in the control mixture was also shown to be active after 12 hours.

Following denaturation of the protein the reaction and control mixtures were extracted with ethyl acetate and the extracts were examined by t.l.c. The extract from the control reaction contained three components which were the same as the most polar and the two least polar of the five components in the extract of the steroid reaction. Consequently all but two components of the latter extract could be eliminated as non-steroidal substances. The major component was the unreacted 5β-androstane-3,17-dione which when developed with sulphuric acid gave a characteristic yellow spot fringed by blue and later changing to a purple spot. The final spot in the extract from the steroid reaction corresponded to the epimeric 3-hydroxy-5β-androstan-17-one standards. All had the same polarity and gave a scarlet spot fringed by blue, which changed to deep purple.

The free and the trimethylsilylated extract containing the reduced steroids was examined by g.l.c. Chromatography of either the free or the trimethylsilylated 3-hydroxy-5β-androstan-17-one standards gave two peaks for each steroid, probably due to thermal elimination. Similar behaviour was

observed when free epiandrosterone and androsterone were chromatographed under the same conditions. However, it was possible to tentatively identify the product (retention times, 5.92 and 7.12 minutes) of the enzymatic reduction of 5β-androstane-3,17-dione as 3β-hydroxy-5β-androstan-17-one (retention times, 5.92 and 7.12 minutes). 3α-Hydroxy-5β-androstan-17-one gave peaks with retention times of 6.40 and 7.36 minutes. The identification of the reduction product as the 3β-alcohol is in accordance with the observations of Waller et al.[216] that LADH is stereospecific for 3β-hydroxy-5β-cholanic acids.

5.1.iii. Attempted reduction of oxymetholone with LADH

Oxymetholone was incubated with LADH and NADH as described for 5β-androstane-3,17-dione except that the reaction was continued for 70 hours, after which time the enzyme was shown to be still active. In contrast to 5β-androstane-3,17-dione which gave a suspension in AMP buffer oxymetholone was completely soluble.

T.l.c. of the product extract after incubation of oxymetholone with the enzyme showed ten components with considerable streaking, indicative of degradation. One spot corresponded to unreacted oxymetholone and one was of the same polarity as 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one. The two least polar components corresponded to the non-steroidal contents of the control extract and the most polar spot was visible under ultraviolet radiation but invisible after sulphuric acid development of the plate. The remaining five spots were more

polar than oxymetholone but none appeared to match either of the 2α-hydroxymethyl-17α-methyl-5α-androstane-3,17-diols.

Oxymetholone and its chemical reduction products were gas chromatographed both as free steroids and as their trimethylsilyl derivatives but in both cases many unidentifiable peaks were obtained. Adhikary[32] also observed that oxymetholone and its metabolites were thermally unstable. Because of the low conversion to reduced products in the enzymatic reaction and the poor gas chromatography of this class of steroids no further information about the structure of the enzymatic reduction products was obtained.

In view of the streaking observed in the t.l.c. of the extract of the enzyme reaction oxymetholone was incubated in a pH 10.5 buffer solution with no LADH or NADH to assess the extent of degradation produced by the alkaline medium. To determine whether the extraction procedure was responsible for degradation one sample was neutralised with very dilute hydrochloric acid after incubation but prior to extraction. However there was no difference between the final extract from this sample and that from a sample which had not been so neutralised. Both contained three components, one of which was oxymetholone, one was less polar and the other more polar. All three components were present in the extract obtained after incubation of the steroid with enzyme but the most polar component from the incubation without enzyme did not correspond to 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one. Consequently, this compound was tentatively identified as a product of the enzymatic reduction of oxymetholone, but the original object of the

experiment, to obtain information concerning the configuration of the 3-hydroxyl group in the most polar metabolite of oxymetholone in man was not fulfilled.

5.1.iv. Attempted reaction of methandrostenolone with LADH

As described in the introduction (section 1, pages 14-16), the $\Delta^{1,4}$-dien-3-one system has been shown to be exceptionally resistant to enzymatic reduction, although reductions to the 3α-hydroxy-4,5β-dihydro-, 3α-hydroxy-4,5α-dihydro- and the 1-ene-3-one-4,5β-dihydro- A-rings have been observed. Methandrostenolone was thus incubated with LADH under the same conditions as those described for oxymetholone to examine whether reduction would take place with this enzyme system.

T.l.c. of the product showed that methandrostenolone and a trace of a slightly more polar component were present. Gas chromatography of the free and the trimethylsilylated extract also showed methandrostenolone with two very small peaks at shorter retention times. However both these peaks were present in chromatograms of the extract from incubation of methandrostenolone in buffer with no LADH or NADH. Consequently, as expected, the $\Delta^{1,4}$-dien-3-one system was resistant to reduction by LADH. The trimethylsilyl derivative of the extract after the enzyme reaction also showed that no 17-epimethandrostenolone was formed.

5.2. Experimental Section

5.2.i. Reaction of 5β-androstane-3,17-dione with liver alcohol dehydrogenase

5β-Androstane-3,17-dione (4.73mg, 16.4 μmole, Sigma Chemical Co.) was ground to a fine powder and transferred to a flask with 2-amino-2-methylpropan-1-ol buffer (AMP, pH 10.5, 0.1M, 20ml). Solutions of β-dihydro nicotinamide adenine dinucleotide (NADH, 24.25mg, 31.6 μmole) in AMP buffer (2.5ml) and horse liver alcohol dehydrogenase (LADH, activity 2.2 units/mg of protein, 1.95mg) in phosphate buffer (pH 7, 1ml) were added to the steroid suspension. The flask was stoppered, shaken to mix the contents and placed in a water bath at 30°, where it was gently agitated. A control, identical but for the omission of the steroid, was set up.

After 12 hours 0.25ml aliquots of the steroid reaction mixture and the control were transferred to separate tubes, diluted with AMP buffer (1ml) and acetaldehyde (0.035ml) was added to each. The tubes were incubated at 30° for 30 minutes, the contents of each were diluted to 20ml, samples were transferred to cuvettes and the ultraviolet spectra recorded. The disappearance of the NADH absorption at 340 nm indicated that the enzyme had remained active throughout the reaction period.

The steroid was isolated by addition of the reaction mixture to ethanol (400ml) which solution was heated to boiling, cooled and filtered. The ethanol was removed by evaporation *in vacuo* to near dryness and water was added. The aqueous phase was extracted twice with ethyl acetate, the combined organic extracts were washed three times with

water, dried over anhydrous magnesium sulphate and evaporated to dryness to give a brown oily residue (9.8mg). Extraction of the control in the same way gave a similar residue (8.2mg).

T.l.c. of both residues showed that the two least polar and the most polar components in the steroid extract corresponded to the only three components in the control. Of the two other components in the steroid product the major was identical in colour and polarity (R_f 0.58) to the starting material and the last spot, small in comparison to that of 5β-androstane-3,17-dione, was of the same polarity (R_f 0.36) and gave the same colour reaction as the epimeric 3-hydroxy-5β-androstan-17-ones. G.l.c. of the steroid extract showed peaks at 7.48 (major), 7.12 and 5.92 minutes. The retention times of the three standard compounds were:- 5β-androstane-3,17-dione, 7.48 minutes, 3β-hydroxy-5β-androstan-17-one, 7.12 and 5.92 minutes and 3α-hydroxy-5β-androstan-17-one, 7.36 and 6.40 minutes. Consequently the product was tentatively identified as 3β-hydroxy-5β-androstan-17-one.

5.2.ii. Attempted reduction of oxymetholone with LADH

Oxymetholone (5.0mg, 15.53 μmole) was treated with NADH (25.95mg) and LADH (3.15mg) as described in the preceding section. The reaction was continued for 70 hours at 30°C. After 68 hours samples of the steroid and control mixtures were tested for enzyme activity by incubation with acetaldehyde. The tests were positive.

Bright yellow residues (12.5mg and 8.3mg) were obtained after extraction of the steroid and control mixtures,

respectively. T.l.c. of both extracts showed that the two least polar components in the steroid extract were the only two components in the control. The steroid extract contained at least eight other components with streaking, indicative of degradation. One of the spots (R_f 0.28) was of the same polarity as 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one.

The reaction was repeated and the same product mixture obtained.

5.2.iii. Examination of the stability of oxymetholone at pH 10.5.

Oxymetholone (5.7mg) was dissolved in AMP buffer (22.5ml) and phosphate buffer (1ml) and incubated at 30° for 72 hours. A second flask containing oxymetholone (5.0 mg) in the same solvent mixture was treated similarly. After incubation the contents of the first flask were neutralised with dilute hydrochloric acid. Both reactions were then extracted as described in section 5.2.i. to give residues (13.3mg and 13.6mg) from the neutralised and un-neutralised samples, respectively. T.l.c. showed that both extracts contained the same three spots, one corresponding to oxymetholone, one less polar and one more polar, all of which were present in the steroid extract after incubation with LADH (section 5.2.ii.). Neither of the extracts obtained after incubation of oxymetholone without enzyme contained a component with the same polarity as 17β-hydroxy-2α-hydroxymethyl-17-methyl-5α-androstan-3-one.

5.2.iv. Attempted reaction of methandrostenolone with LADH.

Methandrostenolone (4.9mg) was treated with NADH (25.0mg) and LADH (3.82mg) as described previously (section 5.2.i.). Two controls, one containing all the ingredients but the steroid and the other containing the steroid but no LADH and NADH were set up. All three flasks were incubated for 72 hours, after which period the activity of the enzyme was confirmed by reaction with acetaldehyde.

The contents of the three flasks were extracted as described in section 5.2.i. to give residues (27.3mg, 24.1mg and 27.1mg) from the steroid + enzyme, enzyme alone and steroid alone mixtures, respectively. T.l.c. of each extract showed two low polarity spots identical to those observed in the control and steroid extracts in the preceding reactions. The major component in both the steroid-containing extracts had the same polarity as methandrostenolone and both showed a trace of a slightly more polar component.

G.l.c. of the extract of the steroid + enzyme incubation showed that the major component had a retention time of 15.56 minutes. Two small peaks at 8.36 and 6.12 minutes were also present. The trimethylsilyl derivative of this extract gave a peak at 14.92 minutes and very small peaks at 9.04 and 6.24 minutes. Standard methandrostenolone trimethylsilyl ether had a retention time of 14.92 minutes in contrast to that of 17-epimethandrostenolone

trimethylsilyl ether, 11.00 minutes. The reaction extract contained no trace of such a peak. The trimethylsilyl derivative of the extract obtained after incubation of methandrostenolone in buffer alone gave the same peaks as the extract from the steroid + enzyme incubation.

The reaction was repeated with similar results.

Bibliography

1. C.D. Kockakian and J.R. Murlin, J. Nutrition, 1935, 10, 437; C.D. Kockakian, Vitamins and Hormones, 1946, 4, 255.

2. G.F. Woods, Reports on the Progress of Applied Chemistry, 1968, 53, 229.

3. E. Eisenberg and G.S. Gordan, J. Pharmac. Exp. Therap., 1950, 99, 38.

4. L.G. Hershberger, E.G. Shipley and R.K. Meyer, Proc. Soc. Exp. Biol. Med., 1953, 83, 175.

5. H.L. Kruskemper, Anabolic Steroids, Academic Press, New York, 1968; 5a, Chapter 4; 5b, Chapter 1; 5c, Chapter 2; 5d, Chapter 5; 5e, Chapter 3.

6. A. Boris, R.H. Stevenson and T. Trmal, Steroids, 1970, 15, 61.

7. D.E. Holtkamp, E.A. Henning and L.F. Mansor, J. Clin. Endocr., 1955, 15, 848.

8. J. van der Vies, Acta Endocr. (Copenhagen), 1965, 49, 271.

9. M.B. Lipsett, Ann. N.Y. Acad. Sci., 1971, 179, 442.

10. A. Bowers, A.D. Cross, J.A. Edwards, H. Carpio, M.C. Calzada and E. Denot, J. Med. Chem., 1963, 6, 156.

11. A. Bernelli-Zazzera, M. Bassi, R. Comolli and P. Lucchelli, Nature, 1958, 182, 663.

12. K. Torizuka, K. Hamamoto, K. Koshiyama, K. Iwai, H. Takayama and T. Miyaka, Metabolism, 1963, 12, 11.

13. K.L. Sydnor, Endocrinology, 1958, 62, 322.

14. C.D. Kockakian, Proc. Soc. Exp. Biol. Med., 1952, 80, 386.

15. A. Segaloff and R.B. Gabbard, Endocrinology, 1960, 67, 887.

16. M.S. de Winter, C.M. Siegmann and S.A. Szpilfogel, Chem. Ind., 1959, 905.

17. P.D. Klimstra, R. Zigman and R.E. Counsell, J. Med. Chem., 1966, 9, 924.

18. E. Vischer, Ch. Meystre and A. Wettstein, Helv. Chim. Acta, 1955, 38, 1502.

19. W.L. Duax, D.A. Norton, S. Pokrywiecki and C. Eger, Steroids, 1971, 18, 525.

20. H.J. Ringold, E. Batres, O. Halpern and E. Necoechea, J. Amer. Chem. Soc., 1959, 81, 427.

21. D.V. Parke, The Biochemistry of Foreign Compounds, Pergamon Press, Oxford, 1968; 21a, pages 3-8; 21b, pages 99 and 172; 21c, page 115; 21d, page 50.

22. M. Stylianou, E. Forchielli, M. Tummillo and R.I. Dorfman, J. Biol. Chem., 1961, 236, 692.

23. M. Stylianou, E. Forchielli and R.I. Dorfman, J. Biol. Chem., 1961, 236, 1318.

24. K. Schubert and K. Wehrberger, Endokrinologie, 1970, 55, 257.

25. C.J.W. Brooks, A.R. Thawley, P. Rocher, B.S. Middleditch, G.M. Anthony and W.G. Stillwell, Advances in Chromatography, Proc. Int. Symp. 6th., 1970, 262, (A. Zlatkis, Ed.).

26. F. Galletti and R. Gardi, Steroids, 1971, 18, 39.

27. A.M. Lawson and C.J.W. Brooks, Biochem. J., 1971, 123, 25P.

28. E. Fingl and D.M. Woodbury, The Pharmacological Basis of Therapeutics, 4th Edn., (L.S. Goodman and A. Gilman, Eds.), Macmillan, New York, 1970.

29. D.S. Layne, T. Golab, K. Arai and G. Pincus, Biochem. Pharmacol., 1963, 12, 905.

30. S. Kamyab, P. Littleton and K. Fotherby, J. Endocr., 1967, 39, 423.

31. J.M. Cooper and A.E. Kellie, Steroids, 1968, 11, 133.

32. P.M. Adhikary, Ph.D. Thesis, University of Edinburgh, 1969.

33. P.M. Adhikary and R.A. Harkness, Biochem. J. 1967, 105, 40P.

34. P.M. Adhikary and R.A. Harkness, Anal Chem., 1969, 41, 470.

35. P.M. Adhikary and R.A. Harkness, Biochem. J., 1969, 112, 30-31P.

36. P.M. Adhikary and R.A. Harkness, J. Chromatogr., 1969, 42, 29.

37. P.M. Adhikary and R.A. Harkness, Acta Endocr. (Copenhagen), 1971, 67, 721.

38. P. Talalay, Physiol. Rev., 1957, 37, 362.

39. G.E. Turfitt, Biochem. J., 1948, 42, 376.

40. K.H. Palmer, J.F. Feierabend, B. Baggett and M.E. Wall, J. Pharmac. Exp. Therap., 1969, 167, 217.

41. C.J.W. Brooks, R.V. Brooks, K. Fotherby, J.K. Grant, A. Klopper and W. Klyne, J. Endocr., 1970, 47, 265.

42. E.L. Rongone and A. Segaloff, Steroids, 1963, 1, 179.

43. E.L. Rongone and A. Segaloff, J. Biol. Chem., 1962, 237, 1066.

44. E.L. Rongone, A. Segaloff, J. Fried and E.F. Sabo, J. Biol. Chem., 1961, 236, 2624.

45. T. Sandor and A. Lanthier, Unpublished progress report, Ciba Ltd., Basle, 1963.

46. F. Kuipers, R.S. Ely and V.C. Kelley, Endocrinology, 1958, 62, 64.

47. S. Burstein, R.I. Dorfman and E.M. Nadel, Arch. Biochem. Biophys., 1954, 53, 307.

48. E.M. Nadel, S. Burstein and R.I. Dorfman, Arch. Biochem. Biophys., 1956, 61, 144.

49. J.C. Touchstone, M. Kasparow and O. Rosenthal, Federation Proc., 1959, 18, 340.

50. J.R. Florini, L.L. Smith and D.A. Buyske, J. Biol. Chem., 1961, 236, 1038.

51. D.L. Berliner and H.A. Salhanick, J. Clin. Endocr., 1956, 16, 903.

52. M.M. Lipman, F.H. Katz and J.W. Jailer, J. Clin. Endocr., 1962, 22, 268.

53. R. Gardi and F. Galletti, Proc. 3rd Meet. Int. Study Group for Steroid Hormones, 1968, 3, 67.

54. K. Schubert and G. Schumann, Endokrinologie, 1970, 56, 172.

55. E. Gerhards, B. Nieuweboer, G. Schulz, H. Gibian, D. Berger and W. Hecker, Acta Endocr. (Copenhagen), 1971, 68, 96.

56. J.A. Gustafsson and B.P. Lisboa, Eur. J. Biochem., 1970, 13, 554.

57. C.H. Gray, M.A.S. Green, N.J. Holness and J.B. Lunnon, J. Endocr., 1956, 14, 146.

58. A. Vermeulen, J. Clin. Endocr., 1956, 16, 163.

59. W.R. Slaunwhite and A.A. Sandberg, J. Clin. Endocr., 1957, 17, 395.

60. D.F. Johnson, E. Heftmann and J.J. Bunim, Proc. Soc. Exp. Biol. Med., 1957, 94, 291.

61. J.J. Bunim, M.M. Pechet and A.J. Bollet, J. Amer. Med. Assn., 1955, 157, 311.

62. S. Burstein, K. Savard and R.I. Dorfman, Endocrinology, 1953, 52, 448; ibid., 1953, 53, 88.

63. S. Roberts and C.M. Szego, Ann. Rev. Biochem., 1954, 24, 543.

64. E. Caspi and M.M. Pechet, J. Biol. Chem., 1958, 230, 843.

65. A.A. Sandberg and W.R. Slaunwhite, J. Clin. Endocr., 1957, 17, 1040.

66. E.M. Glenn, R.O. Stafford, S.C. Lyster and B.J. Bowman, Endocrinology, 1957, 61, 128.

67. J.H.U. Brown and A. Anason, Endocrinology, 1958, 62, 103.

68. H. Schriefers, W. Korus and W. Dirscherl, Acta Endocr. (Copenhagen), 1957, 26, 331.

69. A. Segaloff, R.B. Gabbard and B.T. Carriere, Steroids, 1966, 7, 321.

70. K. Schubert and G. Schumann, Endokrinologie, 1970, 56, 1.

71. K. Schubert, J. Schlegel, H. Groh, G. Rose and C. Horhold, Endokrinologie, 1972, 59, 99.

72. K.J. Ryan, Acta Endocr. (Copenhagen), 35 Suppl., 1960, 51, 697.

73. H. Breuer, Acta Endocr. (Copenhagen), 1962, 40, 111.

74. L. Lutzmann and E. Gerhards, Klin. Woschschr., 1961, 39, 953.

75. J. Booth and J.R. Gillette, J. Pharmac. Exp. Therap., 1962, 137, 374.

76. A.H. Conney, M. Jacobson, K. Schneidman and R. Kuntzman, Life Sciences, 1965, 4, 1091.

77. T.R. Tephley and G.J. Mannering, Mol. Pharmacol., 1968, 4, 10.

78. R. Kuntzman, Ann. Rev. Pharmacol., 1969, 9, 21.

79. R. Kuntzman, M. Jacobson, W. Levin and A.H. Conney, Biochem. Pharmacol., 1968, 17, 565.

80. J.B. Brown, J. Endocr., 1962, 24, 251.

81. S.-G. Lee and C. Chen, Steroids, 1971, 18, 565.

82. K.H. Kimbel, K.H. Kolb and P.E. Schulze, Arzneimittel Forsch., 1962, 12, 223.

83. E.D. Nielson, P.B. Hamilton, M. Riano, D. Rosi and G.P. Peruzzotti, U.S. Patent 3,198,809 (to Sterling Drug Inc.); Chem. Abs., 1965, 63, 14943f.

84. A.J. Manson, R.E. Sjogren and M. Riano, J. Org. Chem., 1965, 30, 307.

85. A.S. Goldman, Endocrinology, 1969, 85, 325.

86. L. Ruzicka and H. Kägi, Helv. Chim. Acta, 1936, 19, 842.

87. K. Miescher and W. Klarer, Helv. Chim. Acta, 1939, 22, 962.

88. F. Sondheimer, O. Mancera, M. Urquiza and G. Rosenkranz, J. Amer. Chem. Soc., 1955, 77, 4145.

89. S.N. Ananchenko, V.N. Leonov, V.I. Zaretskii, N.S. Wulfson and I.V. Torgov, Tetrahedron, 1964, 20, 1279.

90. J. Elks and C.W. Shoppee, J. Chem. Soc., 1953, 241.

91. O.S. Madaeva and F.A. Lur'i, Doklady Akad. Nauk SSSR., 1952, 84, 713; Chem. Abs., 1953, 47, 3326c.

92. T.F. Gallagher and T.H. Kritchevsky, J. Amer. Chem. Soc., 1950, 72, 882.

93. E. Batres, G. Rosenkranz and F. Sondheimer, J. Amer. Chem. Soc., 1955, 77, 4155.

94. G. Wittig, Experientia, 1956, 12, 41.

95. G. Wittig, Angew. Chem., 1956, 68, 505.

96. F. Sondheimer and R. Mechoulam, J. Amer. Chem. Soc., 1957, 79, 5029.

97. E.J. Corey and M. Chaykovsky, J. Amer. Chem. Soc., 1962, 84, 866.

98. R. Greenwald, M. Chaykovsky and E.J. Corey, J. Org. Chem., 1963, 28, 1128.

99. G. Drefahl, K. Ponsold and H. Schick, Chem. Ber., 1965, 98, 604.

100. G. Drefahl, K. Ponsold and H. Schick, East German Patent 37,335; Chem. Abs., 1965, 63, 11677c.

101. N. Sheppard and D.M. Simpson, Quart. Rev., 1952, 6, 1.

102. C. Djerassi, Org. React., 1951, 6, 207.

103. C. Djerassi, R.R. Engle and A. Bowers, J. Org. Chem., 1956, 21, 1547.

104. K. Bowden, I.M. Heilbron, E.R.H. Jones and B.C.L. Weedon, J. Chem. Soc., 1946, 39.

105. L.F. Fieser, J. Amer. Chem. Soc., 1953, 75, 5421.

106. L.H. Zalkow, R. Hale, K. French and P. Crabbé, Tetrahedron, 1970, 26, 4947.

107. H. Budzikiewicz, C. Djerassi and D.H. Williams, Interpretation of Mass Spectra of Organic Compounds, Holden-Day, San Francisco, 1964; 107a, page 54; 107b, page 155.

108. G. von Unruh and G. Spiteller, Tetrahedron, 1970, 26, 3289.

109. G. von Unruh and G. Spiteller, Tetrahedron, 1970, 26, 3329.

110. J.E. Bridgeman, P.C. Cherry, A.S. Clegg, J.M. Evans, Sir E.R.H. Jones, A. Kasal, V. Kumar, G.D. Meakins, Y. Morisawa, E.E. Richards and P.D. Woodgate, J. Chem. Soc. (C), 1970, 250.

111. L.F. Fieser and M. Fieser, Steroids, Reinhold Publishing Corp., New York, 1959; 111a, page 202; 111b, page 519; 111c, page 467.

112. L.F. Fieser and M. Fieser, Reagents for Organic Synthesis, John Wiley and Sons, Inc., New York, 1967; 112a, page 135; 112b, page 911; 112c, page 1179; 112d, page 215; 112e, page 314; 112f, page 315; 112g, page 1140.

113. L. Ruzicka, M.W. Goldberg and E. Hardegger, Helv. Chim. Acta, 1942, 25, 1297.

114. J.R. Moyer and N.C. Manley, J. Org. Chem., 1964, 29, 2099.

115. H.R. Nace and G.L. O'Connor, J. Amer. Chem. Soc., 1951, 73, 5824.

116. E.J. Becker and E.S. Wallis, J. Org. Chem., 1955, 20, 353.

117. D.N. Kirk and M.P. Hartshorn, Steroid Reaction Mechanisms, Elsevier Publishing Co., Amsterdam, 1968; 117a, page 135; 117b, page 29; 117c, page 41; 117d, page 277; 117e, page 269; 117f, page 102; 117g, page 26; 117h, page 36; 117i, page 49; 117j, page 294; 117k, page 99; 117l, page 88; 117m, page 181.

118. S. Bernstein, S.M. Stolar and M. Heller, J. Org. Chem., 1957, 22, 472.

119. G. Muller, J. Martel and C. Huynh, Bull. Soc. Chim. France, 1961, 2000.

120. G. Wittig and U. Schoellkopf, Org. Syntheses, 1960, 40, 66.

121. A.E. Pierce, Silylation of Organic Compounds, Pierce Chemical Co., U.S.A., 1968; 121a, page 3; 121b, page 332; 121c, page 34; 121d, page 35.

122. R.W. Kelly, private communication.

123. M. Makita and W.W. Wells, Anal. Biochem., 1963, 5, 523.

124. E.M. Chambaz and E.C. Horning, Anal. Biochem., 1969, 30, 7.

125. N. Sakauchi and E.C. Horning, Anal. Lett., 1971, 4, 41.

126. Mass Spectrometry, Volume 1, Specialist Periodical Report, The Chemical Society, London, 1971; 126a, R.G. Cooks and G.S. Johnson, page 172; 126b, C.J.W. Brooks, page 300.

127. Rodd's Chemistry of Carbon Compounds, 2nd Edition, Volume 11e, Elsevier Publishing Co., Amsterdam, 1970, page 62.

128. H.-W. Fehlhaber, D. Lenoir and R. Tschesche, Advances in Mass Spectrometry, 1968, 4, 183.

129. K. Biemann and J. Seibl, J. Amer. Chem. Soc., 1959, 81, 3149.

130. V.I. Zaretskii, N.S. Wulfson, V.G. Zaikin, S.N. Ananchenko, V.N. Leonov and I.V. Torgov, Tetrahedron, 1965, 21, 2469.

131. V.I. Zaretskii, N.S. Wulfson, V.G. Zaikin, L.M. Kogan N.E. Voishvillo and I.V. Torgov, Tetrahedron, 1966, 22, 1399.

132. V.I. Zaretskii, N.S. Wulfson, V.G. Zaikin, V.N. Leonov and I.V. Torgov, Tetrahedron, 1968, 24, 2339.

133. V.I. Zaretskii and V.G. Zaikin, Izv. Akad. Nauk. SSSR, Ser. khim., 1969, 8, 1722; Chem. Abs., 1970, 72, 3634g.

134. M.F. Grostic and K.L. Rinehart, J. Org. Chem., 1968, 33, 1740.

135. L.E. Peterson, Chem. Ind., 1962, 264.

136. L. Peterson, Anal. Chem., 1962, 34, 1781.

137. R.H. Shapiro and C. Djerassi, J. Amer. Chem. Soc., 1964, 86, 2825.

138. H. Budzikiewicz, C. Djerassi and D.H. Williams, Structure Elucidation of Natural Products by Mass Spectrometry, Volume 2, Holden-Day, San Francisco, 1964, page 87.

139. S. Popov, G. Eadon and C. Djerassi, J. Org. Chem., 1972, 37, 155.

140. M. Spiteller-Friedmann and G. Spiteller, Org. Mass Spectrom., 1968, 1, 231.

141. J. Diekman and C. Djerassi, J. Org. Chem., 1967, 32, 1005.

142. S.H. Pines, R.A. Firestone, L.Re, M.A. Kozlowski and K. Sletzinger, Steroids,1966, 8, 877.

143. P.E. Shaw, J. Org. Chem., 1966, 31, 2119.

144. S.N. Ananchenko, V.Y. Limanov, V.N. Leonov, V.M. Rzheznikov and I.V. Torgov, Tetrahedron, 1962, 18, 1355.

145. G.E. Mallett, U.S. Patent 3,128,238 (to Eli Lilly and Co.), Chem. Abs., 1964, 61, 1229h; G. Wix, G. Ambrus and K. Albrecht, Hungarian Patent 151,374, Chem. Abs., 1964, 61, 6354e; A. Nobile, U.S. Patent 3,060,092 (to Schering Corp.), Chem. Abs., 1963, 58, 1887d; T. Takahashi, Y. Uchibori and T. Hasegawa, Nippon Nogeikagaku Kaishi, 1962, 36, 67, Chem. Abs., 1964, 61, 8858g.

146. T.H. Stoudt, Advances in Applied Microbiology, Volume 2, (W.W. Umbreit, Ed.), Academic Press, 1960, page 195.

147. Chemical and Biological Aspects of Steroid Conjugation, (S. Bernstein and S. Solomon, Eds.), Springer-Verlag, New York, 1970; S. Bernstein, J.P.Dusza and J.P. Joseph, 147a, page 57; 147b, page 59; P.K. Siiteri, 147c, page 192.

148. S. Burstein and S. Lieberman, J. Amer. Chem. Soc., 1958, 80, 5235.

149. H. Bostrom and B. Wengle, Acta Endocr. (Copenhagen), 1967, 56, 691.

150. S. Burstein and R.I. Dorfman, J. Biol. Chem., 1963, 238, 1656.

151. S. Lieberman, Proceedings of 2nd Int. Cong. Hormonal Steroids, Milan, 1966, page 22.

152. A. Ruokonen, T. Laatikainen, E.A. Laitinen and R. Vihko, Biochemistry, 1972, 11, 1411.

153. K.D. Roberts, L. Bandi, H.I. Calvin, W.D. Drucker and S. Lieberman, Biochemistry, 1964, 3, 1983.

154. H.A. Calvin and S. Lieberman, J. Clin. Endocr., 1966, 26, 402.

155. K.D. Roberts, L. Bandy and S. Lieberman, Biochem. Biophys. Res. Commun., 1967, 29, 741.

156. E.-E. Baulieu, C. Corpéchot, F. Dray, R. Emiliozzi, M. Lebeau, P. Mauvais-Jarvis and P. Robel, Recent Prog. Horm. Res., 1965, 21, 411.

157. R.C. Jenkins and E.C. Sandberg, Methods in Enzymology, 1969, 15, 351.

158. A.E. Sobel, I.J. Drekter and S. Natelson, J. Biol. Chem., 1936, 115, 381.

159. A.B. Roy and P.A. Trudinger, The Biochemistry of Inorganic Compounds of Sulphur, Cambridge University Press, New York, 1970, page 50.

160. M. Levitz, Steroids, 1963, 1, 117.

161. J.P. Joseph, J.P. Dusza and S. Bernstein, Steroids, 1966, 7, 577.

162. P. Baumgarten, Chem. Ber., 1926, 59, 116.

163. E.E. Gilbert, Chem. Rev., 1962, 62, 549.

164. J.P. Dusza, J.P. Joseph and S. Bernstein, Steroids, 1968, 12, 49.

165. R.O. Mumma, Lipids, 1966, 1, 221.

166. R.O. Mumma, C.P. Hoiberg and W.W. Weber, Steroids, 1969, 14, 67.

167. S. Bernstein, J.P. Dusza and J.P. Joseph, Physical Properties of Steroid Conjugates, Springer-Verlag, 1968.

168. F. D'Alo, Farmaco (Pavia) Ed. Sci., 1957, 12, 166.

169. A.B. Roy, Biochem. J., 1956, 63, 294.

170. J.J. Schneider and M.L. Lewbart, J. Biol. Chem., 1956, 222, 787.

171. Y. Nose and F. Lipmann, J. Biol. Chem., 1958, 233, 1348.

172. B. Wengle and H. Boström, Acta Chem. Scand., 1963, 17, 1203.

173. E.-E. Baulieu and R. Emiliozzi, Bull. Soc. Chim. Biol., 1962, 44, 823.

174. R. Emiliozzi, Bull. Soc. Chim. France, 1960, 911.

175. J. McKenna and J.K. Norymberski, J. Chem. Soc., 1957, 3889.

176. Z. Zakrzewski, S. Raczkowska and W. Lewenstein, Przemysl Chem., 1964, 43, 564; Chem. Abs., 1965, 62, 12405g.

177. Z. Zakrzewski, A. Groszkiewicz, S. Raczkowska and M. Malinowska, Acta Pol. Pharm., 1969, 26, 511; Chem. Abs., 1970, 72, 109787z.

178. M. Tohda, N. Kuwana and K. Kawabe, Yakagaku Zasshi, 1964, 84, 900.

179. W. Lewenstein, S. Raczkowska and Z. Zakrzewski, Polish Patent 51,535; Chem. Abs., 1967, 67, 117076d.

180. A.A. Akhren, N.E. Voishvillo and L.E. Kulikova, Izv. Akad. Nauk SSSR, Ser. Khim., 1969, 11, 2625; Chem. Abs., 1970, 72, 77429r.

181. E. Caspi, P.K. Grover, N. Grover, E.J. Lynde and Th. Nussbaumer, J. Chem. Soc., 1962, 1710.

182. N.L. Wendler, Molecular Rearrangements, Part 2 (P. de Mayo, Ed.), Interscience, New York, 1964; 182a, page 1020; 182b, page 1028.

183. J.R. Dyer, Applications of Absorption Spectroscopy of Organic Compounds, Prentice-Hall, Inc., U.S.A., 1965, page 99.

184. F. Tishler and S.M. Brody, J. Pharm. Sic., 1964, 53, 161.

185. E.P. Oliveto, C. Gerold, L. Weber, H.E. Jorgensen, R. Rausser and E.B. Hershberg, J. Amer. Chem. Soc., 1953, 75, 5486.

186. J.G. Moffatt, Oxidation (Techniques and applications in organic synthesis), (R.L. Augustine and D.J. Trecker, Eds.), Volume 2, Marcel Dekker, Inc., New York, 1971; 186a, page 21; 186b, page 38.

187. P. Bladon and W. McMeekin, J. Chem. Soc., 1960, 2191.

188. R.J. Bridgewater and C.W. Shoppee, J. Chem. Soc., 1953, 1709.

189. L. Tokés, R.T. LaLonde and C. Djerassi, J. Org. Chem., 1967, 32, 1020.

190. V. Franzen and H.-E. Driessen, Tetrahedron Lett., No.15, 1962, 661.

191. Parke Davis and Co., Netherlands Appl. Patent 6,514,607; Chem. Abs., 1966, 65, 10641e.

192. D. Bertin and L. Nedelec, Bull. Soc. Chim. France, 1964, 2140.

193. Dictionary of Organic Compounds, Volume 3, Eyre and Spottiswoode Ltd., London, 1965, page 1286.

194. G. Zweifel and H.C. Brown, Org. React., 1963, 13, 28.

195. D.J. Collins, J. Chem. Soc., 1959, 3919.

196. G.R. Pettit, A.K. Das Gupta and R.L. Smith, Can. J. Chem., 1966, 44, 2023.

197. G.H. Douglas, P.S. Ellington, G.D. Meakins and R. Swindells, J. Chem. Soc., 1959, 1720.

198. J. Iriarte, G. Rosenkranz and F. Sondheimer, J. Org. Chem., 1955, 20, 542.

199. M.N. Huffman, U.S. Patent 2,996,524 (to Lasdon Foundation, Inc.); Chem. Abs., 1962, 56, 3546b.

200. H.B. Henbest and M. Smith, J. Chem. Soc., 1957, 926.

201. R.E. Marker and L. Plambeck, J. Amer. Chem. Soc., 1939, 61, 1332.

202. P.D. Klimstra, U.S. Patent 3,169,134 (to G.D. Searle and Co.); Chem. Abs., 1965, 62, 11877a.

203. P.S. Ellington, D.G. Hey and G.D. Meakins, J. Chem. Soc. (C), 1966, 1327.

204. N.S. Bhacca and D.H. Williams, Applications of N.M.R. Spectroscopy in Organic Chemistry, Holden-Day, Inc., San Francisco, 1964; 204a, page 19; 204b, page 95.

205. J.D. Ballantine, Ph.D. Thesis, University of Edinburgh, 1968.

206. L.H. Knox and E. Velarde, J. Org. Chem., 1962, 27, 3925.

207. W.G. Dauben, G.J. Fonken and D.S. Noyce, J. Amer. Chem. Soc., 1956, 78, 2579.

208. E.L. Eliel and M.N. Rerick, J. Amer. Chem. Soc., 1960, 82, 1367.

209. A.S. Dreiding and J.A. Hartman, J. Amer. Chem. Soc., 1953, 75, 939.

210. S. Sloan, D.J. Harvey and P. Vouros, Org. Mass Spectrom., 1971, 5, 789.

211. F. Sondheimer and G. Rosenkranz, U.S. Patent 2,763,670 (to Syntex, S.A.); Chem. Abs., 1957, 51, 5136a.

212. H.C. Brown and R.F. McFarlin, J. Amer. Chem. Soc., 1956, 78, 252.

213. J.A. Gustafsson, B P. Lisboa and J. Sjövall, Eur. J. Biochem., 1968, 6, 317.

214. B. Eckstein and C. Springer, Endocrinology, 1971, 89, 347.

215. R.I. Freudenthal, R. Rosenfeld, C.E. Cook and M.E. Wall, Biochem. Pharmacol., 1971, 20, 2349.

216. G. Waller, H. Theorell and J. Sjövall, Arch. Biochem. Biophys., 1965, 111, 671.

Note on publication

The following papers have been accepted for publication and copies are enclosed.

The identification of 17α-hydroxy-17β-methylandrosta-1,4-dien-3-one as a metabolite of 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one in man. by B.S. Macdonald, P.J. Sykes, P.M. Adhikary and R.A. Harkness, Biochem. J., 1971, 122, 26P.

The identification of 17α-hydroxy-17-methyl-1,4-androstadien-3-one as a metabolite of the anabolic steroid drug 17β-hydroxy-17-methyl-1,4-androstadien-3-one in man. by B.S. Macdonald, P.J. Sykes, P.M. Adhikary and R.A. Harkness, Steroids, 1971, 18, 753.

APPENDIX

THE IDENTIFICATION OF 17α-HYDROXY-17-METHYL-1,4-ANDROSTADIEN-3-ONE AS A METABOLITE OF THE ANABOLIC STEROID DRUG 17β-HYDROXY-17-METHYL-1,4-ANDROSTADIEN-3-ONE IN MAN

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Received: August 3, 1971

ABSTRACT

The more polar of the two major urinary metabolites of methandrostenolone, 17β-hydroxy-17-methyl-1,4-androstadien-3-one, in man has already been identified as 6β-hydroxymethandrostenolone, 6β, 17β-dihydroxy-17-methyl-1,4-androstadien-3-one. The other metabolite has now been identified as the 17-epimer of methandrostenolone,17α-hydroxy-17-methyl-1,4-androstadien-3-one. The compound was isolated from the freely extractable neutral fraction of urine following the administration of 5 mg of the drug to normal men. The relevant chromatographic fractions from thin layer and gas liquid systems were identified by carbon skeleton chromatography. The 17-epimer has been synthesised, details of which are included, and the previously unidentified metabolite was found to be identical with the synthetic compound.

The characterisation of the 17-epimer defines a hitherto apparently unknown biochemical pathway.

INTRODUCTION

The metabolism of the anabolic steroid methandrostenolone (Dianabol) 17β-hydroxy-17-methyl-1,4-androstadien-3-one has been previously studied by Rongone and Segaloff (1). Extraction, after β-glucuronidase hydrolysis, of the urine from a woman with advanced adenocarcinoma of the lung following administration of 4 g of the drug afforded two metabolites which accounted for 7.25% of the dose. 80% of the extracted material was identified

Reprinted from the Proceedings of the Biochemical Society, 17–18 *December* 1970.
Biochem. J., 1971, Vol. 122, No. 1, 26P.]

The Identification of 17α-Hydroxy-17β-methylandrosta-1,4-dien-3-one as a Metabolite of 17β-Hydroxy-17α-methylandrosta-1,4-dien-3-one in Man

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After the administration of the anabolic steroid 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one (methandrostenolone) to man, two major metabolites have been found in the chloroform extract of fresh urine. The least polar metabolite has now been identified as 17α-hydroxy-17β-methylandrosta-1,4-dien-3-one, the 17-epimer of methandrostenolone. The polar metabolite has already been identified as 6β-hydroxymethandrostenolone by Rongone & Segaloff (1963).

The 17-epimer was synthesized from 3β-hydroxyandrost-5-en-17-one by reaction with triphenylmethylphosphonium bromide to form the 17-methylene derivative, which was oxidized and then isomerized to form the 4-en-3-one; this was oxidized with *m*-chloroperoxybenzoic acid to yield 17β-methyl-17α,20-oxidoandrost-4-en-3-one. The oxidoketone was reduced with lithium aluminium hydride to a 3β,17α-diol, which was oxidized and dehydrogenated in one step with dichlorodicyanobenzoquinone to give 17α-hydroxy-17β-methylandrosta-1,4-dien-3-one. This was clearly distinguished from methandrostenolone by its n.m.r. spectrum and melting point, 221°C, which was similar to that of the metabolite isolated by Rongone & Segaloff (1963).

The freely extractable neutral fraction was obtained from a fresh 24h collection of urine from a normal man after oral administration of 5mg of methandrostenolone. The component with the same 'carbon skeleton' as methandrostenolone (Adhikary & Harkness, 1969), which behaved chromatographically like methandrostenolone, was separated by t.l.c. followed by g.l.c. and then subjected to mass spectrometry (A.E.I. MS902). The epimer and metabolite showed a characteristic and similar pattern; for example, the molecular-ion peak at *m/e* 300 was always smaller than the peak at *m/e* 282. This was reversed for methandrostenolone.

No 17-epimer was found in the administered drug, nor was there any detectable conversion during the isolation procedure. It therefore seems justifiable to conclude that the 17-epimer is a metabolite apparently produced by a new pathway, which is relatively important since about 3.5% of an oral dose can be recovered as the epimer.

Adhikary, P. M. & Harkness, R. A. (1969). *Analyt. Chem.* **41**, 470.
Rongone, E. L. & Segaloff, A. (1963). *Steroids*, **1**, 179.

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The analytical techniques which proved of greatest use in distinguishing the 17-epimers in this investigation were n.m.r., g.l.c. and mass spectrometry.

Nuclear Magnetic Resonance Spectroscopy

100 MHz spectra were run in deuteriochloroform solutions using a Varian HA 100 instrument. The spectra of the epimers are identical but for the position of the C-18 methyl resonance which is at 9.07 τ in the spectrum of methandrostenolone and at 9.26 τ in that of its 17-epimer. This difference in chemical shifts is due to the effect of the orientation of the 17-hydroxyl group on the 18-methyl group. Similar observations have been reported by Ananchenko et al. (10).

Gas Liquid Chromatography

G.l.c. was used initially to show that M was a metabolite of methandrostenolone rather than unchanged drug (3). However, this separation was difficult; in later experiments methandrostenolone and authentic 17-epimethandrostenolone were chromatographed on QF-1 and SE-30 columns, separately and as mixtures. Fractionally different retention times were obtained when the compounds were chromatographed separately, methandrostenolone having the longer retention time. Mixtures of equal amounts of the epimers gave fractional separation of the tops of the peaks, whereas mixtures of unequal amounts gave rise only to a single peak. No peak separation could be achieved using OV-1 columns. These steroids were, therefore, only separable on columns of very high resolving power, as in the initial experiments.

The trimethylsilyl ether (TMSE) derivatives of both epimers were prepared according to the method of Makita and Wells (11) and chromatographed on OV-1 columns at 235°C. The retention time of methandrostenolone TMSE derivative relative to methandrostenolone was 1.08 and of 17-epimethandrostenolone TMSE derivative relative to its parent compound was 0.78. The metabolite M was also converted to its TMSE derivative and was similarly chromatographed. The corresponding retention time ratio was 0.78, indicating M to be 17-epimethandrostenolone.

Mass Spectrometry

Mass spectrometry was carried out using an AEI MS 902 instrument operated at 70 e.V. Spectra of microgram samples of metabolite M were obtained by dissolving the entire sample in dry ether (10-20 µl). A syringe was used to transfer a portion (2-3 µl) of this solution to a quartz probe and the solvent was gently evaporated with a hair dryer. This process was repeated several times until sufficient material (ca. 10 µg) had been transferred to the probe, which was then inserted directly into the spectrometer. Spectra obtained in this way

and 3.83 (J = 2 cps), (2-H), 3.92 (J = 2 cps), (4-H), 8.77 (C-19 methyl), 8.82 (C-20 methyl), 9.26 (C-18 methyl); $[\alpha]_D$ + 3° (C = 0.50). Analysis by accurate mass measurement, calc. for $C_{20}H_{28}O_2$ 300.208919, found 300.210430.

Instruments used were: melting points (corrected) - Kofler block; infra red spectra - Unicam S.P. 200 spectrophotometer; n.m.r. spectra Varian HA 100; mass spectra - AEl, MS902; gas liquid chromatography - Perkin-Elmer model 801; optical rotations - measured at 20° in methanol using a Perkin Elmer model 141 automatic polarimeter.

IDENTIFICATION

The less polar metabolite, M, from several studies was pooled until 100 µg was available. However, even after preparative gas liquid chromatography it could not be obtained entirely pure. It therefore became necessary to investigate the efficiency of the various methods of detection available with regard to establishing conclusively the structure of M.

In view of the similarity between their structures, the spectral, chromatographic and other physical behaviour of methandrostenolone and its 17-epimer was examined. Pure methandrostenolone and 17-epimethandrostenolone prepared as previously described were used for this purpose.

The R_f values of the epimers on silica gel thin layer chromatography were identical in all the solvent systems investigated. Both compounds produced an orange-brown spot after development with 5% sulphuric acid in ethanol spray and heating and a blue spot with 8% w/v phosphomolybdic acid in methanol spray. The infra red spectra of the compounds in carbon disulphide solution were very similar except for slight differences in the fingerprint region, the adsorption maximum for the 1,4-dienone carbonyl being at 1665 $cm.^{-1}$

Rongone and Segaloff (1) quoted the melting point of their unidentified metabolite as 222-224°. The melting point of authentic 17-epimethandrostenolone is 221° and that of methandrostenolone is 163°C. Similar differences in the melting points of pairs of 17-epimers are shown by testosterone, m.p. 155° and 17-epitestosterone, m.p. 221° and by methyltestosterone m.p. 164° and 17β-methylepitestosterone, m.p. 182° (8).

The optical rotations of the following pairs of 17-epimeric compounds have been recorded:- methyltestosterone, +76° and 17β-methylepitestosterone, +67° (8) ; 17-methyl-4-androsten-3β,17β-diol, +14°, and 17-methyl-4-androsten-3β, 17α-diol, +8°; methandrostenolone, +12° and 17-epimethandrostenolone, +3°. The differences between the rotations of each pair of epimers are of the same sign and order of magnitude. Thus compounds V and VI and their respective epimers behave as would be expected (9).

ethanol was removed. The steroid was extracted into ether, washed with saturated sodium bicarbonate solution, and water, and finally dried over anhydrous magnesium sulphate. Crystallisation from acetone afforded white needles of III, (0.76 g); m.p. 129-131° (lit. 129-131° [7]); infra red (CS_2) 895, 1655 (17-methylene), 1675 cm^{-1} (carbonyl); n.m.r. ($CDCl_3$) τ 9.16 (C-18 methyl), 8.80 (C-19 methyl), 5.36 (J = 4 cps) (C-17 methylene), 4.27 (J = 6 cps)-(4-H).

17,20-Oxido-21-nor-4-pregnen-3-one (IV) - The enone (III, 0.5 g) was dissolved in methylene chloride (20 ml) and m-chloroperoxybenzoic acid (0.35 g) was added. The reaction was allowed to proceed for 2 hrs at room temperature. Excess peracid was destroyed with 10% sodium sulphite solution until starch-iodide paper remained white when moistened with a drop of the reaction mixture. The solution was neutralised with saturated sodium bicarbonate solution, washed with water and dried over anhydrous magnesium sulphate. Evaporation to dryness and recrystallisation from acetone gave the oxide (0.45 g); m.p. 180-184° (lit. m.p. 179-182°C [7]); infra red (CS_2) 1675 cm^{-1} (carbonyl); n.m.r. ($CDCl_3$) τ 4.28 (4-H), 7.30 (epoxide), 8.82 (C-19 methyl) and 9.14 (C-18 methyl).

17-Methyl-4-androsten-3β,17α-diol (V) - The oxide (IV, 0.45 g) was dissolved in sodium-dried ether and was slowly added to lithium aluminium hydride (0.2 g) in ether (20 ml). The mixture was refluxed for 30 min and excess reagent was destroyed with water. The steroid was extracted into ether, washed with water and dried over anhydrous magnesium sulphate. Evaporation to dryness and recrystallisation from acetone gave the diol (0.36 g); m.p. 225-228°; infra red (CS_2) 3410 cm^{-1} (hydroxyl); n.m.r. ($CDCl_3$) τ 4.72 (4-H), 5.84 (3α-H), 8.82 (C-20 methyl), 8.94 (C-19 methyl), 9,31 (C-18 methyl); $[\alpha]_D$ + 8° (C = 0.18). Analysis by accurate mass measurement, calc. for $C_{20}H_{32}O_2$ 304.240217, found 304.239655.

17α-Hydroxy-17-methyl-1,4-androstadien-3-one (VI) - The diol (V, 0.35 g) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0.57 g) in dry dioxan (20 ml) were refluxed for 6 hr. The solvent was then removed by evaporation, the residue was taken up in benzene and was filtered through a layer of alumina to remove insoluble quinol. The crude product was chromatographed on an alumina column (10 g. Spence type, activity II). Elution with benzene removed three compounds with no carbonyl function, but elution with benzene-chloroform (1:1) removed two polar compounds. Examination by n.m.r. and mass spectrometry identified the less polar compound as 17α-hydroxy-17-methyl-4-androsten-3-one (17β-methylepitestosterone) (0.04 g); m.p. 180-182° (lit. 182° [8]); infra red (CS_2), 1675 (carbonyl), 3410 cm^{-1} (hydroxyl); n.m.r. ($CDCl_3$) τ 4.28 (4-H), 8.81 (C-19 methyl, 8.81 (C-20 methyl), 9.28 (C-18 methyl) and the more polar compound as 17α-hydroxy-17-methyl-1,4-androstadien-3-one (17-epimethandrostenolone)(0.14 g); m.p. 221°; infra red (CS_2), 910, 1636 (alkene), 1665 (carbonyl), 3410 cm^{-1} (hydroxyl): n.m.r. ($CDCl_3$) 2.94 (J = 10 cps), (1-H), 3.73 (J = 2 cps)

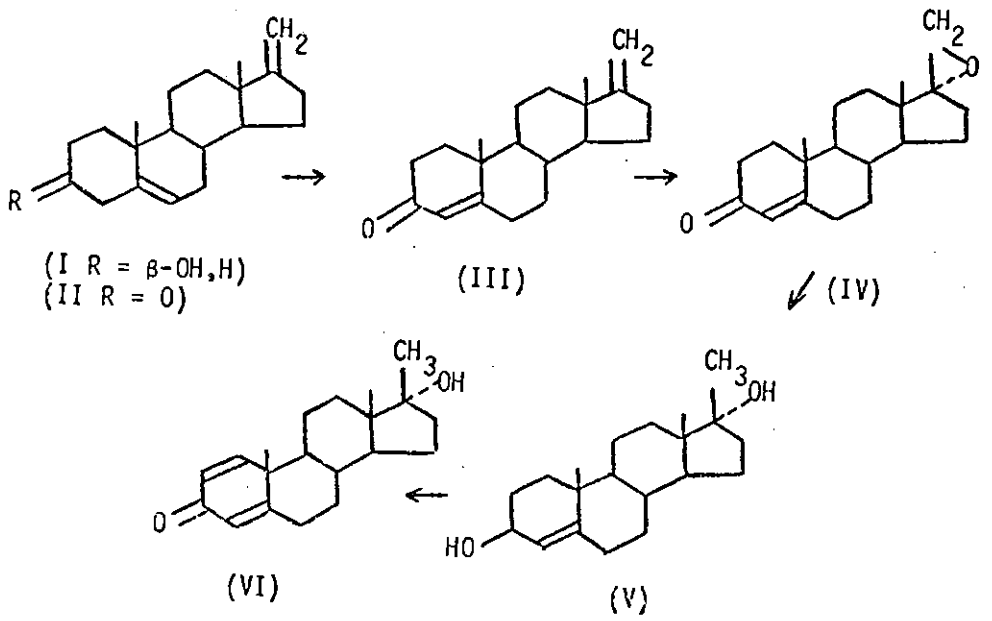

Figure 1

17-Methylene-5-androsten-3-one (II)- The alcohol (I, 3.07 g) was dissolved in ice-cold acetone (215 ml), maintained at 0°, and Jones Reagent (6.14 ml) was added with stirring. After 45 seconds the reaction was quenched with methanol (120 ml) water was added, the organic solvents were removed on a rotary evaporator and the steroid was extracted twice into ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and then with water before being dried over anhydrous magnesium sulphate. Evaporation to dryness yielded a crude product (2.83 g) which after crystallisation from ethanol gave a mixture of II and III, (0.898 g); m.p. 109-117°C; infra red (CS_2) 895, 1655 (17-methylene), 1675 (carbonyl, III), 1715 cm^{-1} (carbonyl, II).

17-Methylene-4-androsten-3-one (III).- The mixture of enones (II and III, 0.85 g) from the previous stage was dissolved in ethanol (50 ml) and anhydrous oxalic acid (0.2 g) was added. The solution was held at 60°C for 30 mins, water was added and the

(positive fractions). These fractions were then purified by further chromatography on thin layers of silica gel G and finally by preparative gas liquid chromatography. Additional confirmation of the carbon skeleton of the compound or compounds in these fractions was again obtained by gas chromatography after high temperature catalytic reduction.

The More polar of the two metabolites showed the same chromatographic behaviour as 6β-hydroxymethandrostenolone. The major metabolite behaved similarly to methandrostenolone on thin layer chromatography, but a slightly shorter retention time on gas chromatography (SE-30 or QF-1) indicated that the compound was not merely unchanged drug.

PREPARATION OF 17-EPIMETHANDROSTENOLONE

The preparation of 17α-hydroxy-17-methyl-4-androsten-3-one (17β-methylepitestosterone) has been described by Sondheimer *et al.* (7), using pregnenolone acetate as starting material. The synthesis of 17-epimethandrostenolone here described is essentially the same although different reagents were used and the starting material was 3β-hydroxy-5-androsten-17-one (dehydroisoandrosterone).

Experimental

17-Methylene-5-androsten-3β-ol (I).- Triphenylmethylphosphonium bromide (25.0 g) and potassium *t*-butoxide : *t*-butanol (1:1 complex, 13.04 g) were dissolved in dry dimethylsulphoxide (80 ml) under nitrogen with gentle warming. A solution of 3β-hydroxy-5-androsten-17-one (4.04 g) in dimethylsulphoxide (80 ml) was added. The reaction mixture was maintained at 80°C for 1hr, diluted with ice and 10% sodium chloride solution and extracted with ether. The ether layers were combined and washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness *in vacuo* to give crude product (5.09 g) heavily contaminated with triphenylphosphine oxide. Recrystallisation from methanol yielded pure I, (3.17 g); m.p. 134-135° (lit. 133-134°C [7]): infra red (CS_2) 895, 1655 (17-methylene), 3450 cm^{-1}(3-OH); n.m.r. ($CDCl_3$) τ9.20 (C-18 methyl), 8.98 (C-19 methyl), 5.36 (J = 4 cps) (C-17 methylene), 4.64 (J = 6 cps) (6-H).

as 6β-hydroxymethandrostenolone 6β, 17β-dihydroxy-17-methyl-1,4-androstadien-3-one. The structure of the second metabolite was not established but on the basis of the evidence available Rongone and Segaloff suggested that it was an isomer of methandrostenolone rather than an oxygenated product.

Adhikary and Harkness have studied the metabolism of small doses of methandrostenolone in normal man (2,3). They succeeded in recovering 5% of the dose as two major metabolites from the freely extractable neutral fraction of the urine. High temperature catalytic reduction with gas chromatography of the hydrocarbon products ('carbon skeleton chromatography' [4,5]) was used to locate the metabolites and to quantify them. The two compounds occurred in the ratio 2:1, the minor of the two being identified as 6β-hydroxymethandrostenolone (2). The 17-epimer of methandrostenolone 17α-hydroxy-17-methyl-1,4-androstadien-3-one has now been synthesised and has been shown to be identical with the major urinary metabolite (M) of methandrostenolone in man (6).

ISOLATION AND PURIFICATION

In each study, the normal therapeutic dose of one 5 mg tablet of methandrostenolone was administered to a male volunteer in good health, and the total urine output of the following 25 hr was collected. Extraction of the urine with chloroform gave the 'free fraction'. The extracts were chromatographed on silica gel G coated plates using chloroform-methanol 19:1 (v/v) as mobile phase. Chromatographic fractions were collected as eluates from 1 cm bands; these were halved, evaporated to dryness and one set of residues dissolved in 0.1 ml of ethanol. 10 µl of this solution from each fraction was used for high temperature catalytic reduction at 180-190°C using 1-3% w/w platinum catalyst. The reduction products were analysed by gas chromatography using SE-30 or NGA columns with a hydrocarbon standard derived from the drug by the same method. From these results, chromatographic fractions were selected which contained the same skeleton as the drug

showed that several compounds were generally present in the metabolite samples even after repeated preparative thin layer chromatography. It was established that the mass spectra of synthetic methandrostenolone and its 17-epimer were unaffected by gas chromatography of the compounds prior to spectrometry. Consequently, metabolite samples were purified by preparative gas chromatography, collected in glass tubes and transferred to the spectrometer probe as already described.

Prior to mass spectrometry of M is was necessary to compare the spectra of pure methandrostenolone Fig. 2(a) and authentic 17-epimethandrostenolone Fig. 2(c). The base peak of both compounds is m/e = 122 (12). The presence of this ion establishes the 1,4-dienone structure of the A-ring, since it must arise by B-ring fragmentation with the transfer of two protons to the charged species, Fig. 3 (13).

Under the conditions encountered within the mass spectrometer 17-epimethandrostenolone readily dehydrates to give a peak at m/e = 282 (M^+-H_2O) which is large compared with the molecular ion, M^+ at m/e = 300. It also gives a peak at m/e = 267 ($M^+-H_2O-CH_3$). Both of these peaks are present in the spectrum of methandrostenolone but are unimportant compared with M^+ and the ion at m/e = 242 which arises from fragmentation across the D-ring. It can be seen from these results that, although both epimers give rise to peaks at the same m/e values the intensities can be used to determine which epimer is present (6). Ananchenko et al. (10) obtained similar results in their study of the mass spectra of 17-epimeric oestranes and 19-nortestosterones and the corresponding D-homo-17a-epimeric compounds.

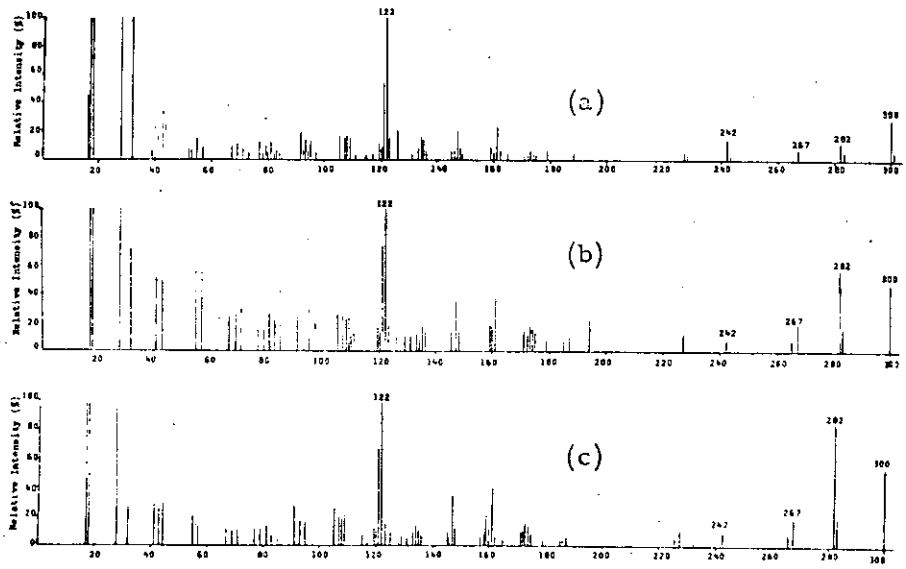

Figure 2

The mass spectrum of the metabolite M after purification by preparative gas chromatography is shown in Fig. 2(b). This spectrum adds further support to the earlier conclusions that M is not merely unchanged drug and verifies the assumption that it is 17-epimethandrostenolone.

For further identification of M the TMSE derivatives of the authentic epimers and M were subjected to mass spectrometry.

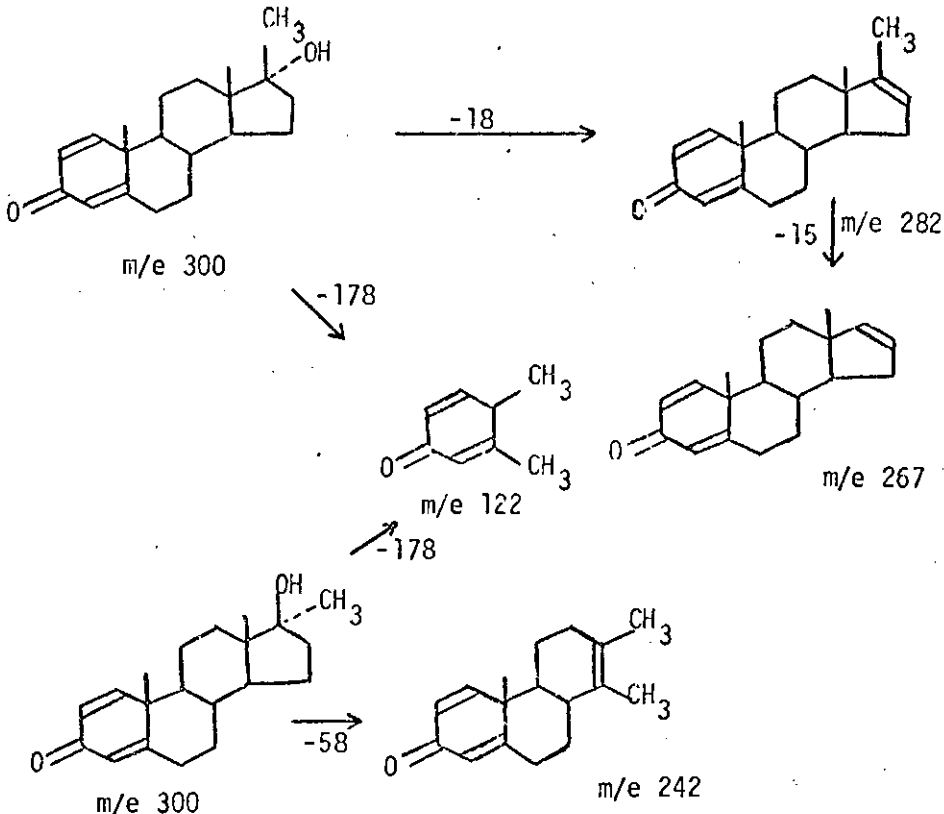

Figure 3

All three gave similar spectra with the molecular ion, M^+ at m/e = 372, indicating the successful formation of the derivative. The base peak is at m/e = 143, the origin of which is shown in fig. 4 (12). The only other ion of importance is that at m/e = 282.

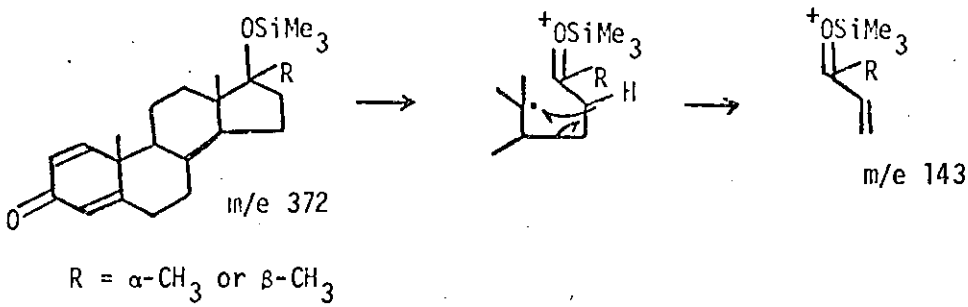

Figure 4

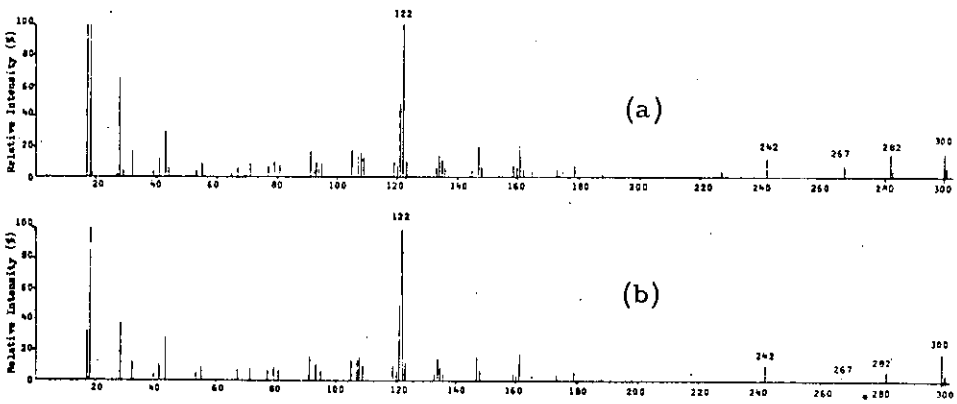

Figure 5

Examination of Commercial Methandrostenolone

Because less than 5% of administered methandrostenolone can be recovered as 17-epimethandrostenolone it seemed advisable to check that tablets of commercial methandrostenolone do not contain a small quantity of the epimer. Accordingly, ten 5 mg tablets of methandrostenolone (Dianabol, Ciba) were ground to a fine powder and extracted with benzene. The insoluble carrier material was removed by filtration and the solvent was removed on a rotary evaporator. Crystals, which had a melting point identical with that of pure methandrostenolone were obtained. Gas liquid chromatography of the TMSE derivative of the drug obtained from tablets showed no 17-epimer.

Fig.5(a) shows the mass spectrum of a synthetic mixture containing 95% pure methandrostenolone and 5% 17-epimethan-

drostenolone. The spectrum of methandrostenolone extracted from tablets is shown in fig.5(b). It is identical with the spectrum of pure methandrostenolone, fig.2(a), and differs from fig.5(a). No epimer was detectable after extracting methandrostenolone from water and subsequent thin layer chromatography during which the samples were exposed to light. The compound has already been shown to be unaltered after gas chromatography. Since the 17-epimer was not present in the starting material, nor was it produced by the isolation procedures, the 17-epimethandrostenolone isolated from urine after the administration of methandrostenolone must be of biochemical origin.

DISCUSSION

Proof that a major metabolite of methandrostenolone in normal man is 17-epimethandrostenolone raises the question as to how it arises. The epimerisation of secondary hydroxyl groups is well known chemically (14) whereas epimerisation of the tertiary 17-hydroxyl group is unknown (15). The epimerisation of secondary steroid alcohols has previously been reported in biochemical systems especially when there is an adjacent oxygen function which can assume an oxo-form (16) but such reactions generally constitute minor metabolic pathways. Biochemical epimerisation of tertiary alcohols, to our knowledge, has not been previously described.

The metabolism of methandrostenolone proceeds quite differently from that of 17α-methyltestosterone (17) where the 4(5)-double bond and the 3-oxo group are both reduced. In addition, there is no evidence of epimerisation at C-17. These findings suggest that the A-ring of methandrostenolone is more stable to the usual enzymatic conversions but may somehow facilitate the epimerisation at C-17. Similar facts

to these have also been observed by Schubert *et al.* (18) in their work on the metabolism of the 4-chloro analogue of methandrostenolone (Oral-Turinabol). These workers have isolated from the freely extractable fraction an unidentified metabolite present in relatively large amounts which may well be the 17-epimer of Oral-Turinabol.

The isolation of 17-epimethandrostenolone from the freely extractable fraction rather than the conjugated 'glucuronide' fraction of urine is unusual; little or in some cases no metabolites were obtained from urine after β-glucuronidase hydrolysis. In addition to the inhibition of glucuronyl transfer to the 17β-hydroxyl group by the 17α-methyl group the structure of the A-ring probably further reduces conjugation of the 17-hydroxyl group; this is consistent with findings of Hsia *et al.* (19) who found that 17β-hydroxy-17-methyl-1,4-androstadien-3-one was four times more effective than 17β-hydroxy-17-methyl-4-androsten-3-one in inhibiting conjugation of o-aminophenol *in vitro*.

The mechanism of formation of the 17-epimer is at present being studied.

ACKNOWLEDGEMENTS

We are grateful to Professor L.G. Whitby and Professor J.I.G. Cadogan for their encouragement, to Mr. A.M. Torrance and Mr. D. Thomas for technical assistance, to Dr. A. Segaloff for a supply of 6β-hydroxymethandrostenolone and to Ciba for the pure methandrostenolone used in this work.

REFERENCES

1. Rongone, E.L., and Segaloff, A., STEROIDS, 1, 179, (1963).

2. Adhikary, P.M., and Harkness, R.A., BIOCHEM.J., 112, 30P (1969).

3. Adhikary, P.M., Ph.D. Thesis, University of Edinburgh, 1969.

4. Adhikary, P.M., and Harkness, R.A., ANALYT.CHEM., 41, 470 (1969).

5. Adhikary, P.M., and Harkness, R.A., J.CHROMATOG., 42, 29 (1969).

6. Macdonald, B.S., Sykes, P.J., Adhikary, P.M., and Harkness, R.A., BIOCHEM.J., 122, 26P (1971).

7. Sondheimer, F., Mancera, O., Urquiza, M., Rosenkranz, G., J.AM.CHEM.SOC., 77, 4145 (1955).

8. Fieser, L.F., and Fieser, M., STEROIDS, Reinhold Publishing Corp., 1959, p. 519.

9. Klyne, W., DETERMINATION OF ORGANIC STRUCTURES BY PHYSICAL METHODS (Eds. Braude, E.A., Nachod, F.C.), 1955, p. 78.

10. Ananchenko, S.N., Leonov, V.N., Zaretskii, V.I., Wulfson, N.S., and Torgov, I.V., TETRAHEDRON, 20, 1279 (1964).

11. Makita, M., and Wells, W.W., ANALYT.BIOCHEM., 5, 523 (1963).

12. Brooks, C.J.W., Thawley, A.R., Rocher, P., Middleditch,B.S., Anthony, G.M., and Stillwell, Wanda G., ADVANCES IN CHROMATOGRAPHY, 1970, Editor A Zlatkis, Chromatography Symposium, University of Houston, Houston 1970, p. 262.

13. Peterson, L., ANALYT.CHEM., 34, 1781 (1962).

14. Chemistry of Carbon Compounds, IID, STEROIDS (Ed. Coffey), 1970, pages 39 and 48.

15. Chemistry of Carbon Compounds, IID, STEROIDS (Ed. Coffey), 1970, pages 271 to 276.

16. Raud, H.R. and Hobkirk, R., BIOCHEM.J., 103, 724, (1967).

17. Rongone, E.L., and Segaloff, A., J.BIOL.CHEM., 237, 1066, (1962).

18. Schubert, K., and Wehrberger, K., ENDOKRINOLOGIE, 55, 257, (1970).

19. Hsia, D.Y.Y., Riabov, S., and Dowden, R.M., ARCH.BIOCHEM. BIOPHYS., 103, 181 (1963).