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The Stereospecific Removal of a C-19 Hydrogen Atom in Oestrogen Biosynthesis

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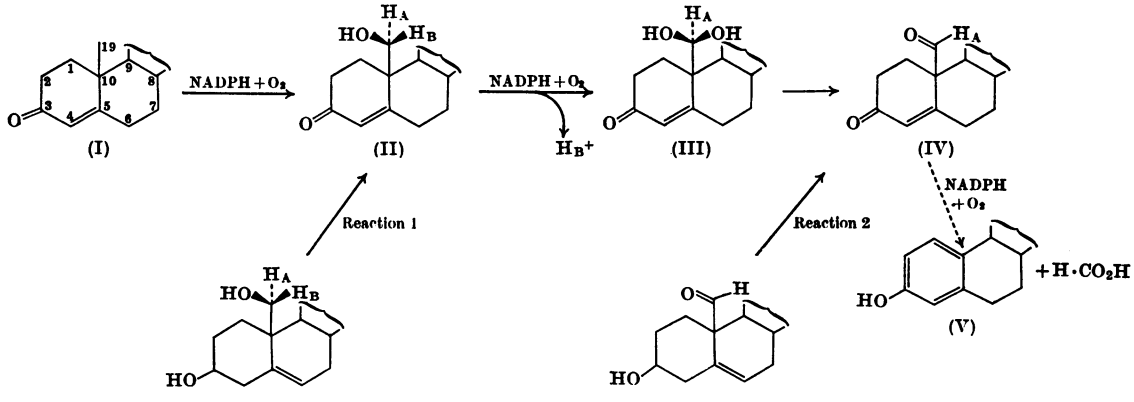
1. The synthesis of a number of 19-substituted androgens is described. 2. A method for the partially stereospecific introduction of a tritium label at C-19 in 19-hydroxyandrost-5-ene-3β,17β-diol was developed. The 19-^{3}H-labelled triol produced by reduction of 19-oxoandrost-5-ene-3β,17β-diol with tritiated sodium borohydride is tentatively formulated as 19-hydroxy[(19-*R*)-19-^{3}H]androst-5-ene-3β,17β-diol and the 19-^{3}H-labelled triol produced by reduction of 19-oxo[19-^{3}H]-androst-5-ene-3β,17β-diol with sodium borohydride as 19-hydroxy[(19-*S*)-19-^{3}H]-androst-5-ene-3β,17β-diol. 3. In the conversion of the (19-*R*)-19-^{3}H-labelled compound into oestrogen by a microsomal preparation from human term placenta more radioactivity was liberated in formic acid (61·6%) than in water (38·4%). In a parallel experiment with the (19-*S*)-19-^{3}H-labelled compound the order of radioactivity was reversed: formic acid (23·4%), water (76·2%). 4. These observations are interpreted in terms of the removal of the 19-*S*-hydrogen atom in the conversion of a 19-hydroxy androgen into a 19-oxo androgen during oestrogen biosynthesis. 5. It is suggested that the removal of C-19 in oestrogen biosynthesis occurs compulsorily at the oxidation state of a 19-aldehyde with the liberation of formic acid.

One of the processes involved in the biosynthesis of an oestrogen from an androgen of the type (I) is the removal of the 19-methyl group. Studies directed towards an understanding of the mechanism of elimination of C-19 have shown that under the conditions of oestrogen biosynthesis both 19-hydroxyandrostenedione (II) (Meyer, 1955; Ryan, 1959; Hayano, Longchampt, Kelly, Gual & Dorfman, 1960; Longchampt, Gual, Ehrenstein & Dorfman, 1960; Breuer & Grill, 1961; Morato, Hayano, Dorfman & Axelrod, 1961; Axelrod & Goldzeiher, 1962; Wilcox & Engels, 1965) and 19-oxoandrostenedione (IV) (Akhtar & Skinner, 1968) are formed. The efficient conversion of compounds (II) and (IV) into oestrone (V) is also well documented (Akhtar & Skinner, 1968). These observations are interpreted in terms of a sequence of reactions of the type outlined in Scheme 1 for the



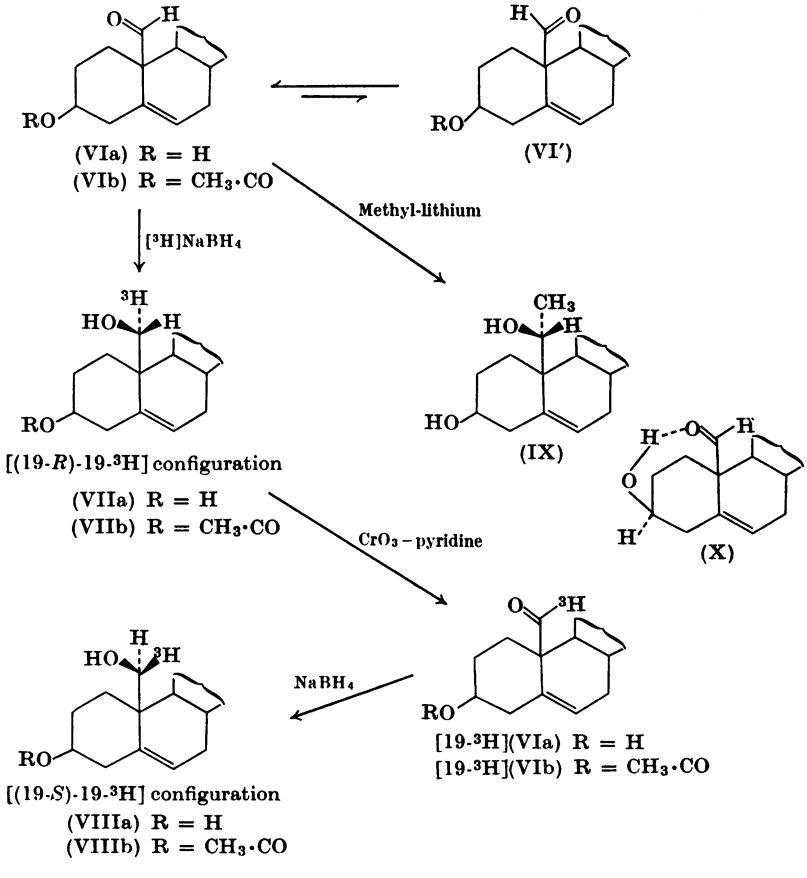
Scheme 1. Reactions 1 and 2 refer to the conversion, in two steps, of the 3β-hydroxy-5-ene system into the 3-oxo-4-ene system. H_A represents the 19-*R*-hydrogen atom; H_B represents the 19-*S*-hydrogen atom.

removal of C-19 in oestrogen biosynthesis. The requirement for NADPH and oxygen in the formation of the 19-oxo compound (IV) from the corresponding hydroxy compound (II) (Akhtar & Skinner, 1968) is particularly noteworthy and this has been attributed to the involvement of a dihydroxy intermediate of the type (III).

Work on reactions at 'non-activated' carbon atoms has made available a number of compounds (Akhtar & Barton, 1962; Akhtar & Barton, 1964; Kalvoda, Huesler, Ueberwasser, Anner & Wettstein, 1963) required for the stereospecific introduction of a tritium label at C-19 in 19-hydroxy androgens. We have now synthesized these compounds and have used them for studying the enzymic mechanisms of some of the reactions of Scheme 1. To highlight the oxidation and stereochemical status of C-19 a non-systematic steroid nomenclature is used in this paper.

RESULTS AND DISCUSSION

Synthesis of (19-*R*)-19-^{3}H-*labelled and* (19-*S*)-19-^{3}H-*labelled*-19-*hydroxy androgens.* The two hydrogen atoms at C-19 in 19-hydroxyandrost-5-ene-3β,17β-diol (structure of type VIIa and VIIIa, Scheme 2) are chemically equivalent and therefore the introduction of a label stereospecifically into either one of them should offer a special challenge. We have, however, taken advantage of the observation of Caspi & Wicha (1966), who showed that the reaction of methyl-lithium with the 19-aldehyde group in the compound (VIb) gave predominantly a single alcohol for which the structure (IX) was proposed. The formation of the alcohol with the stereochemistry shown in (IX) may be rationalized if it is assumed that the methyl carbanion generated from methyl-lithium attacks the carbonyl group from the α-side of the steroid molecule, the carbonyl



Scheme 2.

group existing predominantly in the form (VI) rather than the alternative extreme form (VI'). If the stereoelectronic factors responsible for directing the attack of methyl-lithium on the aldehyde (VIb) are maintained in the reaction of the same compound (VIb) or a similar compound (VIa) with tritiated sodium borohydride, then the resulting primary alcohol should contain the tritium atom predominantly in the *R*-configuration, as shown in the structure (VIIa and VIIb) in Scheme 2. The aldehyde (VIa) containing hydroxyl groups at positions 3β and 17β was reduced with tritiated sodium borohydride to furnish the radioactive triol (VIIa); the latter compound, as noted below, contained radioactivity associated predominantly with one of the two C-19 hydrogen atoms. By analogy with the reaction of methyl-lithium on the aldehyde (VIb) we tentatively formulate the compound obtained from the reaction of tritiated sodium borohydride with the aldehyde (VIa) as being predominantly 19-hydroxy[(19-*R*)-19-^{3}H]-androst-5-ene-3β,17β-diol (VIIa). Similarly the reaction of the aldehyde (VIb), which contained a 3β,17β-diacetoxy function, with tritiated sodium borohydride gave the compound (VIIb), which was hydrolysed with methanolic potassium hydroxide to the triol (VIIa). The enzymic experiments described in the next section will show that the steric distribution of tritium in the triol (VIIa) produced by the latter method was almost identical with that obtained in the triol (VIIa) produced by the direct reduction of compound (VIa) with tritiated sodium borohydride. This suggests that the nature of the 3β-substituent does not influence the steric course of the reduction. The configuration assigned to the alcohol (IX) by Caspi & Wicha (1966) and, by implication, to the compound (VIIa) in the present work would require that, of the two orientations (VI) and (VI') for the aldehyde group, the arrangement (VI) is favoured. The explanation that the observed stereospecificity is due to hydrogen-bonding between the 3β-hydroxy group and the 19-carbonyl group (X), favouring the conformation (VI), may be rejected since the change from a 3β-hydroxyl group to a 3β-acetoxyl group exercised no influence on the steric course of reduction. It is therefore suggested that the conformation (VI) may be favoured because of the superior overlap of the π orbitals that may be achieved in the former case owing to the transoid arrangement of the 5,6-double bond and the carbonyl group. The participation of the 5,6-double bond on the photochemical reactivity of the 19-aldehyde group has previously been observed (Akhtar, 1965). The present work suggests that the 5,6-double bond may also influence the ground-state conformation of the aldehyde group in the compounds (VIa) and (VIb).

When the diacetoxy alcohol (VIIb) was oxidized with chromium trioxide in pyridine the radioactive aldehyde (19-tritiated VIb) was obtained in good yield. The latter was then hydrolysed with methanolic 5% potassium hydroxide to the compound (19-tritiated VIa), which on reduction with sodium borohydride furnished the triol (VIIIa). This contained radioactivity associated predominantly with the opposite hydrogen to the compound (VIIa) and therefore may be tentatively formulated as 19-hydroxy[(19-*S*)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIIa).

Biological conversion of the 3β-hydroxy-5-ene system into a 3-oxo-4-ene system. The two crucial compounds (VIIa) and (VIIIa) having been made available, we were in a position to study the absolute stereochemistry of hydrogen elimination in the conversion of the 19-hydroxy androgen (II) into the corresponding 19-oxo compound by human term-placental microsomal preparation.

To appreciate the main theme developed below it is, however, necessary to include some supplementary comments. It is to be noted that the microsomal preparation used in the present work for oestrogen biosynthesis includes an additional enzyme system that converts 3β-hydroxy-5-ene compounds into the corresponding 3-oxo-4-ene compounds (reactions 1 and 2, Scheme 1). Another feature requiring comment is the fact that whether the precursors of oestrogen contain a 17-oxo or a 17β-hydroxy group it seems from our work that the predominant product is the 17-oxo-group-containing oestrone (V). This observation suggests that during oestrogen biosynthesis with the placental microsomal preparation the 17β-hydroxy group is readily oxidized to a carbonyl group. The oxidation–isomerization sequence represented by reaction 1 and reaction 2 in Scheme 1 is particularly advantageous since it allows a convenient generation *in situ* of the (19-*R*)-19-^{3}H-labelled and (19-*S*)-19-^{3}H-labelled compounds of structure (II) from the corresponding compounds (VIIa) and (VIIIa); such a conversion would be cumbersome if it had to be performed chemically.

Biological conversion of the (19-*R*)-19-^{3}H-*labelled and* (19-*S*)-19-^{3}H-*labelled* 19-*hydroxy compounds into oestrone.* The sequence proposed in Scheme 1 would require that in the conversion of the alcohol (II) into oestrone (V) one of the two hydrogen atoms at C-19 of (II) will be liberated as a proton (II $\rightarrow$ III, Scheme 1) and the other hydrogen atom released with formic acid (IV $\rightarrow$ V, Scheme 1). When the (19-*R*)-19-^{3}H-labelled 19-hydroxy compound (VIIa) was incubated under aerobic conditions with the microsomal preparation and NADPH about 28–35% of the original radioactivity was recovered in the acid-volatile fraction. That the percentage radioactivity found in the acid-volatile fraction in fact measures the conversion of compounds of types

Table 1. *Liberation of radioactivity as formaldehyde, formic acid and water in the biological conversion of (19-R)-19-^{3}H-labelled and (19-S)-19-^{3}H-labelled 19-hydroxyandrost-5-ene-3β,17β-diol (VIIa and VIIIa) and 19-oxo[19-^{3}H]androst-5-en-3β-ol-17-one into oestrone*

Incubations were carried out as described in the Experimental section. All incubations contained steroid substrate (0·66 μmole), $NADP^+$ (5·2 μmoles), glucose 6-phosphate (26·1 μmoles) and glucose 6-phosphate dehydrogenase (0·5 unit) for the generation of NADPH. The compound (VIIa) in Expt. 1a was prepared by reduction of 19-oxoandrost-5-ene-3β,17β-diol with tritiated $NaBH_4$ and designated as (19-R)-19-^{3}H. The compound (VIIIa) in Expt. 1b was prepared by reduction of 19-oxo[19-^{3}H]androst-5-ene-3β,17β-diol (VIa) with $NaBH_4$ and designated (19-S)-19-^{3}H. The same two compounds used in Expts. 2a and 2b were prepared by a different sequence of reactions. The compound (VIIa) in Expt. 2a was prepared by reduction of 19-oxoandrost-5-ene-3β,17β-diol diacetate (VIb) with tritiated $NaBH_4$ and subsequent hydrolysis. The compound (VIIIa) in Expt. 2b was prepared by reduction of 19-oxo[19-^{3}H]androst-5-ene-3β,17β-diol diacetate (VIb) with $NaBH_4$ and subsequent hydrolysis.

Expt. no.	Substrate	10^5 × Radioactivity in substrate (c.p.m.)	10^5 × Total radioactivity c.p.m.	Radioactivity recovered in acid-volatile fraction (% of total): In formaldehyde	In formic acid	In water
1a	19-Hydroxy[(19-R)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIa)	20·0	5·60	—	60	40
			6.82	—	64	36
1b	19-Hydroxy[(19-S)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIIa)	12·0	3·48	1	26	73
			3·14	—	20	80
2a	19-Hydroxy[(19-R)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIa)	8·03	1·58	1	59	40
			1·60	—	57	43
2b	19-Hydroxy[(19-S)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIIa)	4·34	0·90	—	16	84
			1·06	—	21	79
3	19-Oxo[19-^{3}H]androst-5-ene-3β-ol-17-one (VIc)	2·01	1·66	—	96	4
			1·55	1	92	7

(VIIa) and (VIIIa) into oestrone was shown by a parallel experiment in which 35–40% conversion of 19-hydroxy[16-^{3}H]androst-5-en-3β-ol-17-one into oestrone was achieved. The volatile fraction, on further fractionation, gave approx. 60% of the radioactivity in formic acid and 40% in water (Expt. 1a, Table 1). In a parallel experiment when the (19-S)-19-^{3}H-labelled 19-hydroxy compound (VIIIa) was treated similarly, approx. 75% of the radioactivity was recovered in water and 25% in formic acid (Expt. 1b, Table 1). The above experiments were repeated with the compounds (VIIa) and (VIIIa) prepared by another sequence of reactions [reduction of the 19-oxo diester (VIb) and then hydrolysis to the 3β,17β,19-triol] and the results showed close agreement (compare Expt. 1a with Expt. 2a and Expt. 1b with Expt. 2b; Table 1).

The compounds (VIIa) and (VIIIa) are chemically identical but from their methods of synthesis we expected that the labelled hydrogen atoms at C-19 in these compounds would possess predominantly opposite stereochemical orientations. This expectation was borne out by the fact that, on being converted into oestrogen, the compound (VIIa) gave almost 2·5 times the radioactivity in formic acid as did the compound (VIIIa).

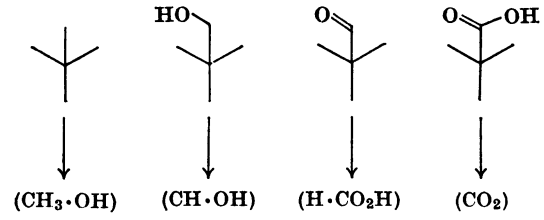
Further confirmation for the opposite orientations of labelled hydrogen atoms at C-19 in these compounds came from the measurement of radioactivity in water. Thus in the conversion of the (19-S)-19-^{3}H-labelled compound (VIIIa) into oestrogen about 80% of the radioactivity of the volatile fraction was recovered in water, whereas the (19-R)-19-^{3}H-labelled compound gave only 40% of such radioactivity in water. Scheme 1 suggests that the radioactivity in water results from the release of a proton in the conversion (II) → (IV). The higher percentage radioactivity obtained in water from the (19-S)-19-^{3}H-labelled compound (VIIIa) shows that it is the 19-S-hydrogen atom that is eliminated in the conversion (II) → (IV). That the 19-R-hydrogen atom of (II) in oestrogen biosynthesis is eliminated with C-19 as formic acid is established by the fact that a greater amount (60%) of radioactivity is found in formic acid from the (19-R)-19-^{3}H-labelled compound (VIIa) than from the (19-S)-19-^{3}H-labelled compound (VIIIa), which gave only 25% of volatile radioactivity in formic acid. The absolute configurations allotted to the compounds (VIIa) and (VIIIa), although based on sound analogue, must be considered tentative until their configurations can be confirmed by an independent method.

Conversion of 19-*oxo*[19-^{3}H]*androst*-5-*en*-3β-ol-

17-one into oestrone. Quantitative conversion of compounds (VIIa) and (VIIIa) into oestrogen (V) should produce equal and opposite amounts of radioactivity in formic acid and water; the values in Table 1 are not exactly equal. This small discrepancy, although not affecting the main conclusions drawn in this work, did, however, require further scrutiny.

When 19-oxo[19-^{3}H]androst-5-en-3β-ol-17-one was converted into oestrone by the microsomal preparation (Expt. 3, Table 1) about 80% of the original radioactivity was recovered in the acid-volatile fraction, approx. 95% in formic acid and 5% associated with water. This result suggests that some activity from the 'C-19 aldehyde group' may be released into water under the general conditions of incubation and preparation. We are therefore tempted to suggest that in Expts. 1a and 2a (Table 1) some of the 'formic acid' radioactivity has been converted into water, thus artificially decreasing the percentage of radioactivity in formic acid and increasing the percentage of radioactivity in water. A similar conversion of the 'formic acid' radioactivity into water will tend artificially to decrease the formic acid and increase the water radioactivities in Expts. 1b and 2b (Table 1). Although these considerations do not allow the precise estimation of the degree of stereospecificity of the two samples of 19-^{3}H-labelled 19-hydroxy compounds (VIIa) and (VIIIa) to be made, they do, however, convincingly prove that these compounds contained the tritium label predominantly in opposite orientation.

Oxidation state of C-19 *and the removal of* C-19. The fact that in the conversion of the 19-oxo-[19-^{3}H]androst-5-en-3β-ol-17-one and the 19-^{3}H-labelled 19-hydroxy compounds (VIIa and VIIIa) into oestrone the radioactivity associated with a C_1 unit was found entirely in formic acid suggests that the cleavage of the C-10–C-19 bond in the biosynthesis occurs at the oxidation state of an aldehyde (Scheme 3 shows the C_1 compounds formed by the cleavage of a C–C bond at various states of oxidation). The view that the removal of C-19 might have occurred at the state of a 19-hydroxy intermediate of the type (II) and that formic acid was formed subsequently by an enzymic or non-enzymic oxidation of the resulting formaldehyde may be rejected by the following considerations. The two hydrogen atoms of formaldehyde are equivalent chemically as well as biochemically. If the removal of C-19 in oestrogen biosynthesis occurred at the level of a 19-hydroxy intermediate then formaldehyde derived either from the (19-*R*)-19-^{3}H-labelled compound (VIIa) or from the (19-*S*)-19-^{3}H-labelled compound (VIIIa) will contain radioactivity distributed equally between its two hydrogen atoms. The further oxidation of formaldehyde must therefore give the same distribution of radioactivity in formic acid and water whether the precursor was compound (VIIa) or compound (VIIIa).



Scheme 3.

In conclusion, the current status of oestrogen biosynthesis may be summarized as follows. The conversion of an androgen of the type (I) into oestrone involves at least two successive hydroxylations. The first hydroxylation results in the formation of a 19-hydroxy compound of the type (II) and the second hydroxylation gives a 19-oxo compound of the type (IV). The latter is probably formed through the intermediacy of a diol of the type (III). In the conversion of the 19-hydroxy compound (II) into the 19-oxo compound (IV) it is the 19-*S*-hydrogen atom that is removed. It is emphasized that the removal of C-19 in oestrogen biosynthesis must occur at the oxidation level of an aldehyde, thus liberating formic acid. The reactions occurring in ring A in the further conversion of the aldehyde (IV) into oestrone (V) are less well understood. Indirect evidence, however, suggests that another hydroxylated intermediate may intervene between compounds (IV) and (V) (Townsley & Brodie, 1968; Akhtar & Skinner, 1968).

EXPERIMENTAL

Infrared spectra were determined on a Unicam SP. 200 spectrometer and ultraviolet spectra on a Unicam SP. 800 spectrometer. Melting points were determined on a Gallenkamp Melting Point Apparatus and are uncorrected. All the compounds described below gave the expected i.r. and u.v. spectra. For preparative (2mm.) and analytical (0·4mm.) t.l.c. silica gel preparations H_F254 and G_F254 respectively (E. Merck A.-G., Darmstadt, Germany) were used. All radioactivity measurements were taken on a Beckman Liquid Scintillation System, model CPM 200, with Butyl-PBD [2-(4-*tert.*-butylpheny)-5-(4-biphenylyl)-1-oxa-3,4-diazole] from CIBA (A.R.L.) Ltd., Duxford, Cambs., as scintillant in toluene (8g./l.) with up to 50% (v/v) of methanol for counting aqueous solutions. All samples were corrected for quenching with respect to a standard sample of [19-^{3}H]cholest-5-en-3β-ol acetate, either by external or by internal standardization. The counting efficiency was about 40–50%. $NADP^+$ (disodium salt), NAD^+ and glucose 6-phosphate dehydrogenase were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Dehydroepiandrosterone acetate

(androst-5-en-3β-ol-17-one acetate) was supplied by Henly and Co., New York, N.Y., U.S.A. All other chemicals (Reagent and Biochemical Grades) were obtained from BDH Chemicals Ltd., Poole, Dorset.

Preparation of human term-placental microsomes and incubation procedure. Human term placentae were cooled to 0° within 5min. of delivery and dissected and homogenized within 45min. of delivery. Microsomal preparations were made as described by Ryan (1959) and stored at −°16 as portions equivalent to approx. 30g. wet wt. of tissue in 2·5ml. of 0·05M-sodium phosphate buffer, pH7·1. Each incubation mixture contained 2·5ml. of 0·05M-sodium phosphate buffer, pH7·1, 0·66μmole of steroid substrate dissolved in 0·1ml. of methanol, 5·2μmoles of $NADP^+$, 0·5unit of glucose 6-phosphate dehydrogenase, 26μmoles of glucose 6-phosphate and 2·5ml. of the microsomal suspension in buffer, and was incubated at 37° for up to 2hr. Incubations were terminated and the steroids extracted as described by Wilcox & Engels (1965) and separated by t.l.c. as described by Akhtar & Skinner (1968).

Analysis of formate, formaldehyde and water from incubations of 19-^{3}H-*labelled* 19-*oxygenated androgens.* The incubations were terminated by adding formaldehyde (39mg.), formic acid (72mg.) and 0·5ml. of orthophosphoric acid (sp.gr. 1·75). The reaction mixture was then transferred to a round-bottomed flask and the volatile fraction distilled into a liquid-N_2 trap by freeze-drying. A 0·1ml. portion of the condensate was counted in methanol–Butyl-PBD in toluene (3:8, v/v) (11ml.) and the remaining solution divided into two parts.

The first half was tritrated with 0·1M-NaOH, with phenolphthalein as indicator, to form the sodium salt of formic acid and test the recovery of formic acid, 100% recovery being equivalent to 7·8ml. of 0·1M-NaOH; all recoveries were between 97 and 102%. After titration the total volume was measured accurately and 6ml. of this solution was freeze-dried, and 0·1ml. of the condensate was counted as before. To the residue of sodium formate 2ml. of water was added and the solution was again freeze-dried to remove traces of tritiated water. The residue of sodium formate was dissolved in 3ml. of water and a 0·1ml. sample of the solution was counted.

The second portion of the acid-volatile distillate was analysed for formaldehyde by addition of 40ml. of dimedone reagent as described by Gabriel & Ashwell (1965). The complex was allowed to form a precipitate for 1hr. in the cold, the precipitate was filtered off, washed with water and crystallized twice from methanol–water. A 10–20mg. sample of the complex was weighed accurately and counted in 8ml. of Butyl-PBD in toluene.

Preparation of 19-*hydroxy- and* 19-*oxo-androst-5-ene compounds.* (*a*) 19-Hydroxyandrost-5-en-3β-ol-17-one 3-acetate. 5α-Bromo-6β,19-epoxyandrostan-3β-ol-17-one acetate (Akhtar & Barton, 1964) (1g.) was added to a suspension of zinc dust (6g.) in acetic acid–water (20:1, v/v) (21ml.) and the mixture was stirred at 20° for 1hr. The zinc was filtered off, water was added and the solution extracted into methylene chloride or chloroform. The organic extract was washed sequentially with water and aqueous saturated $NaHCO_3$ soln. and then dried over anhydrous Na_2SO_4. The product crystallized from methylene chloride–light petroleum (b.p. 60–80°), m.p. 155°. 19-Hydroxyandrost-5-en-3β-ol-17-one was prepared as follows. The 3-acetoxy compound (50mg.) in methanolic 5% (w/v) KOH (2ml.) was heated to 50° and then left at room temperature for 2hr. The solution was neutralized with 1M-acetic acid, processed as above and crystallized from chloroform–diethyl ether–light petroleum (b.p. 60–80°).

(*b*) 19-Hydroxyandrost-5-ene-3β,17β-diol 3,17-diacetate. This compound was prepared as above from 5α-bromo-6β,19-epoxyandrostan-3β,17β-diol diacetate (Kalvoda *et al.* 1963) and crystallized from diethyl ether–light petroleum (b.p. 60–80°), m.p. 145–147°. 19-Hydroxyandrost-5-ene-3β,17β-diol was produced from the corresponding 3,17-diacetoxy compound by hydrolyis as described above and crystallized from methanol–water, m.p. 225–228° (decomp.).

(*c*) 19-Oxoandrost-5-en-3β-ol-17-one acetate. 19-Hydroxyandrost-5-en-3β-ol-17-one 3-acetate (100mg.) in pyridine (3·75ml.) was added to a suspension of CrO_3 (150mg.) in pyridine (3·75ml.) and the mixture left at 20° for 4hr. Five such reactions were pooled and extracted with diethyl ether. Pyridine was removed from the ether extract by repeated washing with an ice-cold solution of 0·5M-HCl. The organic extract was then processed as described above. The product was crystallized from diethyl ether–light petroleum (b.p. 60–80°) and then recrystallized from methanol–water, m.p. 133–135°. 19-Oxoandrost-5-en-3β-ol-17-one was prepared as described above from the corresponding 3-acetate by hydrolysis and crystallized from chloroform–light petroleum (b.p. 60–80°).

(*d*) 19-Oxoandrost-5-ene-3β,17β-diol diacetate (VIb). This compound was produced by oxidation of the corresponding 19-hydroxy compound with CrO_3–pyridine as above, crystallized from diethyl ether–light petroleum (b.p. 60–80°) and recrystallized from methanol, m.p. 135–136°. 19-Oxoandrost-5-ene-3β,17β-diol (VIa) was prepared by hydrolysis of the corresponding 3,17-diacetoxy compound as above and crystallized from methanol–water, m.p. 169°.

Preparation of 19-*hydroxy- and* 19-*oxo-androst-4-ene compounds.* (*a*) 5α-Bromo-6β,19-epoxyandrostan-3,17-dione. 5α-Bromo-6β,19-epoxyandrostan-3β-ol-17-one acetate (Kalvoda *et al.* 1963) (1g.) was refluxed for 1hr. in methanol–water–K_2CO_3 (90:10:1, v/v/w) (100ml.). Most of the methanol was evaporated off under vacuum, the residue tipped into ice–water (150ml.), the precipitated steroid filtered off and the filtrate extracted with methylene chloride. The precipitate was added to the methylene chloride extract, and the solution was dried over Na_2SO_4, filtered and evaporated to an oil. The oil was dissolved in acetone (20ml.) and oxidized with a slight excess of Jones reagent (Bowden, Heilbron, Jones & Weedon, 1946). Excess of Jones reagent was removed with methanol and the supernatant decanted from the precipitated chromous salts. The steroid crystallized on dropwise addition of water and cooling. The crystals of this compound, which decomposed at room temperature, were stored at −16°.

(*b*) 6β,19-Epoxyandrost-4-ene-3,17-dione. 5α-Bromo-6β,19-epoxyandrostane-3,17-dione (1g.) was refluxed with methanolic 5% (w/v) potassium acetate (100ml.) for 1hr. The solution was extracted with methylene chloride, processed as above and crystallized from diethyl ether–light petroleum (b.p. 60–80°), m.p. 181°.

(*c*) 19-Hydroxyandrost-4-ene-3,17-dione (II). 5α-Bromo-6β,19-epoxyandrostane-3,17-dione (100mg.) or 6β,19-epoxyandrost-4-ene-3,17-dione (100mg.) was added to a suspension of zinc dust (1·0g.) in acetic acid–water (20:1, v/v) (8·0ml.) and the mixture stirred under reflux for 8min.

Additional zinc dust (1·0g.) was added with 2 drops of 12 M-HCl and stirred for a further 6 min. Five such reaction mixtures were pooled, extracted with chloroform, processed as above and crystallized from chloroform–light petroleum (b.p. 60–80°), m.p. 178°.

(*d*) 19-Oxoandrost-4-ene-3,17-dione (IV). 19-Hydroxyandrost-4-ene-3,17-dione (100mg.) was oxidized with CrO_3–pyridine as describe above but for 12–18hr. The product was extracted into ether and processed as other 19-oxo androgens. The 19-oxo compound (IV) was purified by preparative t.l.c. (2mm.) developed in chloroform–methanol–diethyl ether (95:2:4, by vol.), R_F 0·5–0·6, and eluted with diethyl ether–methanol. The solution was used as a chromatographic standard ($\lambda_{max.}$ 244nm., ϵ 18000). The structure was proved as described by Akhtar & Skinner (1968).

Preparation of tritiated 19-hydroxy and 19-oxo androgens. (*a*) 19-Hydroxy[(19-*R*)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIa). (i) A 100mg. sample of the 19-oxo compound (VIb) was dissolved in methanol (5ml.) that had been previously distilled from $NaBH_4$ to remove any oxidant impurities. The solution was cooled to 0° and approx. 1mg. of tritiated $NaBH_4$ (1mc/mg.) and 0·1ml. of methanolic 5% (w/v) KOH were added to the reaction mixture, which was then allowed to reach 20° over 30min. Excess of non-radioactive $NaBH_4$ (50mg.) was added and the the mixture was left for the completion of the reduction for a further 30min. Excess of $NaBH_4$ was decomposed with acetic acid and the solution extracted into chloroform and processed as described above. After removal of the chloroform under vacuum the oil was treated with methanolic KOH to hydrolyse the 3- and 17-acetoxy groups and processed as described for the non-radioactive compound (specific radioactivity $4{\cdot}157\times10^6$ c.p.m./mg.). (ii) The compound (VIa) (50mg.) was reduced as described for preparation (*a*) with tritiated $NaBH_4$ and crystallized after extraction with chloroform from methanol–water (specific radioactivity $1{\cdot}00\times10^7$ c.p.m./mg.).

(*b*) 19-Oxo[19-^{3}H]androst-5-en-3β-ol-17-one acetate. Non-radioactive 19-oxoandrost-5-en-3β-ol-17-one acetate (50mg.) was reduced as described above with tritiated $NaBH_4$. The crude product was then oxidized with CrO_3–pyridine as in previous reactions but for a period of 18hr. The product was purified by preparative t.l.c. (2mm.) with chloroform for development, R_F 0·6–0·7, and crystallized from diethyl ether–light petroleum (b.p. 60–80°) (specific radioactivity $6{\cdot}22\times10^6$ c.p.m./mg.). 19-Oxo[19-^{3}H]-androst-5-en-3β-ol-17-one was prepared from the corresponding 3-acetoxy compound by hydrolysis as above and crystallized from diethyl ether–light petroleum (b.p. 60–80°) (specific radioactivity $1{\cdot}027\times10^6$ c.p.m./mg.).

(*c*) 19-Oxo[19-^{3}H]androst-5-ene-3β,17β-diol diacetate (VIb). The (19-*R*)-19-^{3}H-labelled 19-hydroxy compound (VIIb) was oxidized with CrO_3–pyridine as described above for 4hr., processed as described above and crystallized from methanol–water to give compound (VIb) (specific radioactivity $9{\cdot}4\times10^6$ c.p.m./mg.). 19-Oxo[19-^{3}H]androst-5-ene-3β,17β-diol (VIa) was prepared by hydrolysis of the corresponding 3,17-diacetoxy compound (VIb) as above and crystallized from methanol–water (specific radioactivity $7{\cdot}8\times10^6$ c.p.m./mg.).

(*d*) 19-Hydroxy[(19-*S*)-19-^{3}H]androst-5-ene-3β,17β-diol (VIIIa). (i) The 19-^{3}H-labelled 19-oxo compound (VIb) was reduced as in the production of the (19-*R*)-19-^{3}H-labelled 19-hydroxy compound (VIIa) but with non-radioactive $NaBH_4$. The product was processed and hydrolysed as before with methanolic KOH and the (19-*S*)-19-^{3}H-labelled trihydroxy compound crystallized from methanol–water (specific radioactivity $7{\cdot}65\times10^6$ c.p.m./mg.).

(ii) The 19-^{3}H-labelled 19-oxo compound (VIa) was reduced with $NaBH_4$ as described above and crystallized from methanol–water (specific radioactivity $6{\cdot}00\times10^6$ c.p.m./mg.).

(*e*) 19-Hydroxy[16-^{3}H]androst-5-en-3β-ol-17-one. 19-Hydroxyandrost-5-en-3β-ol-17-one 3-acetate (50mg.) was treated with methanolic 5% (w/v) KOH containing 0·1ml. of tritiated water (1c/ml.) for 12hr. The product was processed as before, washed repeatedly with water to remove tritiated water, and crystallized from chloroform–light petroleum (b.p. 60–80°) (specific radioactivity $1{\cdot}4\times10^7$ c.p.m./mg.).

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