Mechanistic Consideration of P-450 Dependent Enzymic Reactions: Studies on Oestriol Biosynthesis

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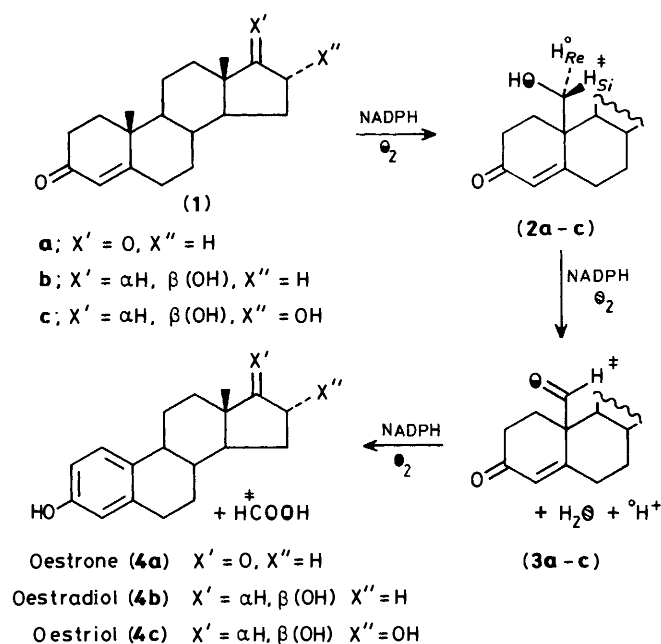
Methods for the synthesis of 19-hydroxy and 19-oxo derivatives of 16α-hydroxytestosterone [(**11b**) and (**12b**) respectively] have been developed. These compounds were labelled with ^{18}O, ^{2}H, and ^{3}H at C-19 and also with ^{3}H at C-17. The conversion of [17α-^{3}H]-16α,19-dihydroxytestosterone (**11c**) into oestriol (**4c**), using human placental aromatase was demonstrated in good yield and it was shown that in this process the 19-oxo compound (**3c**) is involved as an intermediate. The use of 16α,19-hydroxytestosterone, labelled with ^{3}H predominantly in the H_{Si} position, led to the conclusion that in oestriol biosynthesis the step, $-CH_2OH \longrightarrow -CHO$, is accompanied by the loss of H_{Re} and in the overall process the C-19 is ejected as HCOOH. On conducting experiments with either $^{18}O_2$ or substrate containing ^{18}O at C-19 it was shown that, in the conversion of 16α-hydroxy-19-oxotestosterone into oestriol, an atom of oxygen from O_2 is incorporated into the formate. These features are similar to those already established for the corresponding biosynthesis of oestrone/oestradiol from androstenedione/testosterone. Our previous postulate that in oestrogen biosynthesis the same enzyme is involved in the hydroxylation reaction, $-CH_3 \longrightarrow -CH_2OH$, and in the conversion, $-CH_2OH \longrightarrow -CHO$, as well as in the final cleavage of the C-10–C-19 bond is further developed. Attention is drawn to the fact that, if cytochrome P-450 dependent reactions are viewed to occur *via* a radical mechanism, then a concept can be developed which unifies the wide variety of transformations catalysed by this group of enzymes. The diversity of reactions would then arise from the alternative mode of decomposition of radical species by one or a combination of the following processes: (a) hydrogen abstraction; (b) disproportionation; (c) fragmentation; and (d) association of radicals.

The mechanism by which the aromatic ring-A of the redox pair comprising oestrone-oestradiol (**4a**) and (**4b**) is biosynthesized from androgens (**1**), has been the subject of extensive investigations in recent years[1–8] and since the initial finding that oestrogens are synthesized from androgens in human placenta,[2] the mechanism of the aromatisation reaction has been largely elucidated.[8] It is generally accepted that this process is carried out by a cytochrome P-450 dependent enzyme system.[3] It is now known that the first step in the conversion [(**1a**)→(**2a**), Scheme 1] which requires NADPH plus O_2 and involves the hydroxylation[4] of C-19, occurs with overall retention of stereochemistry, to form the androstenedione (**2a**).[5] The second oxidative step again requires NADPH and O_2 and results in the loss[6] of H_{Re} of compound (**2a**) to produce the 19-aldehyde (**3a**).[1a] The latter is subsequently aromatised with the consumption of the 'third' mole of O_2 and NADPH ejection of C-19 as formic acid.[7] In this last transformation, one oxygen atom from the 'third' mole of O_2 is incorporated into the formic acid.[8] This specific finding has eliminated several possible mechanistic pathways for the C-10–C-19 bond cleavage step and has focussed attention upon one possibility.[9] To further evaluate this possibility, it is important to establish whether the chemical process shown in Scheme 1 represents a general phenomenon by studying other examples of the same generic reaction.

Oestriol (**4c**) is the most abundant oestrogen produced during human pregnancy.[10] Although some oestriol is produced by the hydroxylation of oestrone-oestradiol in the foeto-placental unit, the bulk of oestriol is biosynthesized *de novo* from 16α-hydroxy androgens.[11] Despite its physiological significance, the latter process has not been subjected to mechanistic scrutiny. This neglect is primarily due to the difficulty of synthesis and of suitably labelling the putative intermediates of the pathway.

The overall similarity of oestriol production to that of oestrone-oestradiol suggests that 19-hydroxy and 19-oxo



Scheme 1. Pathway for the biosynthesis of oestrogens

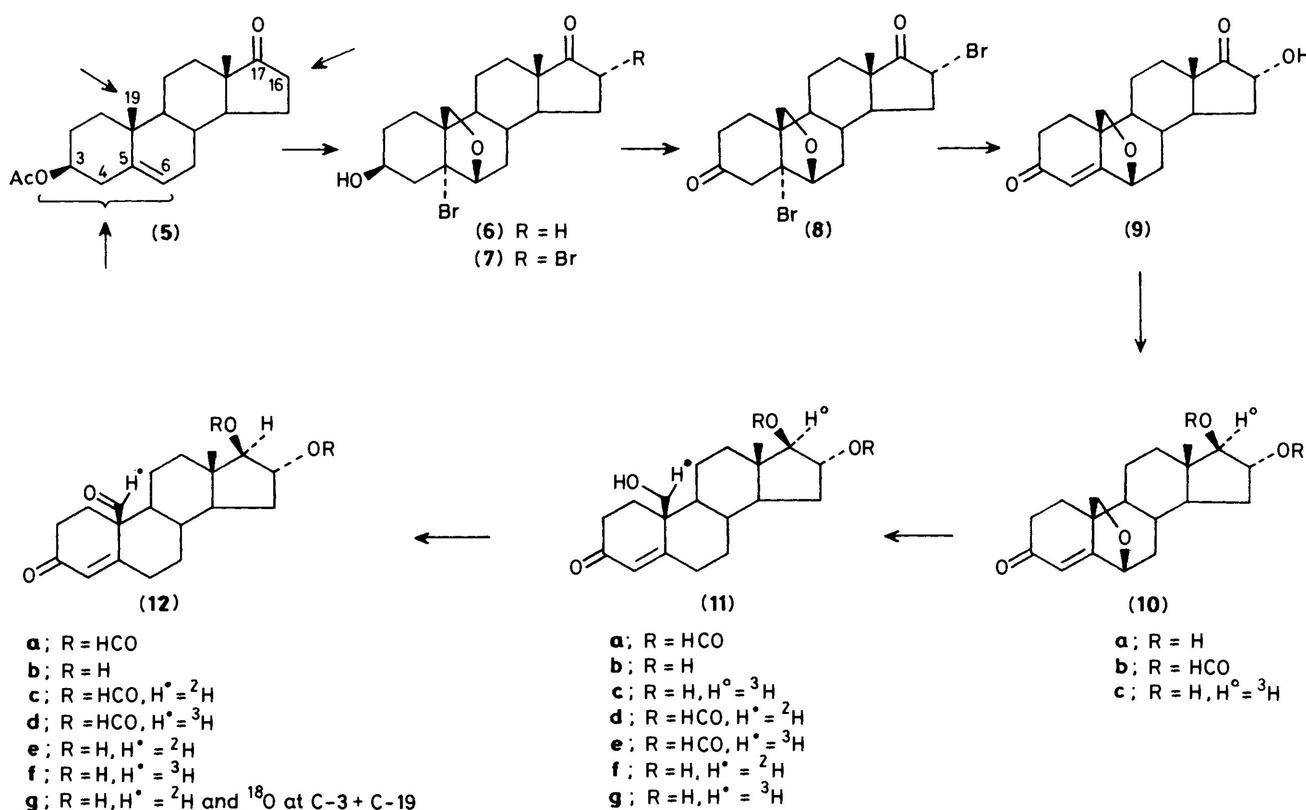
derivatives of 16α-hydroxyandrostenedione and of 16α-hydroxytestosterone (**2c**) and (**3c**) may be involved in an intermediary role. The problem of the synthesis of the compounds in the latter series has now been solved and, after appropriate labelling, these compounds have been used to study the chemistry of the individual steps involved in oestriol biosynthesis. In particular, the origin and fate of various oxygen atoms at C-19 during the final C-10–C-19 bond cleavage step has been delineated. The results obtained in the present paper,

when taken in conjunction with other recent studies on hydroxylation reactions, have prompted us to develop one of the frequently considered proposals into a hypothesis unifying the mechanisms of a variety of transformations catalysed by P-450 dependent enzyme systems. A part of the work has already been published in a preliminary form.[12]

Results and Discussion

1. *Chemical Synthesis. Preparation of* 16α-*Hydroxy*-19-*oxygenated Derivatives of Testosterone* (**11b**) *and* (**12b**).—The starting material for the synthesis of 16α-hydroxy-19-oxygenated derivatives of androstenedione and testosterone was dehydroisoandrosterone acetate (DIA acetate) (**5**). The projected synthesis required the modifications at centres shown by arrows in structure (**5**). They are: conversion of the 3β-hydroxy-5-ene system into a ring-A conjugated ketone, hydroxylation at C-16, functionalisation of C-19 to produce the corresponding 19-hydroxy or 19-oxo derivative, and in the case of 17β-hydroxy compounds, reduction of the 17-carbonyl group. While established methods exist for carrying out the individual conversions, the challenge was to perform the desired reactions at each centre without disturbing the others. Many permutations of the available reactions were tried and whilst the selectivity problems proved insurmountable in the 17-oxo series, the required modifications were successfully achieved with steroids containing a 17β-hydroxy group, permitting the synthesis of the testosterone derivatives (**11b**) and (**12b**). The first stage in the synthesis was the conversion of DIA acetate (**5**) into the 6,19-epoxide (**6**)[13] followed by functionalisation of C-16 by bromination.[14] The acid-stability of the 16α-bromo-17-ketone system in (**7**) was exploited for oxidation of the 3β-hydroxy group of (**7**) to the corresponding diketone (**8**) using Jones reagent.[15] The latter (**8**), when subjected to treatment with two equivalents of potassium carbonate under carefully controlled conditions, underwent elimination of the 5α-bromo group as HBr and the displacement of the 16α-bromo functionality by a hydroxy group to yield (**9**). Previous work[16] has established that the displacement reaction at C-16 occurs with overall retention of stereochemistry. Attempts to open the 6,19-epoxy ring of (**9**) with zinc led to the elimination of the 16α-hydroxy group, so laboriously introduced, regenerating the parent methylene group at C-16. This is the main reason why the 16α-hydroxy-19-oxygenated derivatives of androstenedione have not yet been prepared. This complication was overcome by performing the reductive opening of the 6,19-epoxy ring after the conversion of the 17-carbonyl function into the corresponding hydroxy group. The selective reduction, by sodium borohydride, of the 17-carbonyl group in the presence of a ring-A conjugated system is known to occur smoothly with simpler molecules (androstenedione and 16α-hydroxyandrostenedione). It was, however, found that the selectivity was greatly diminished in the borohydride reduction of the diketone (**9**); presumably the ring-strain arising from the presence of a 6,19-epoxy ring enhanced the reactivity of the 3-ketone. Notwithstanding this, conditions were established to produce the desired diol (**10a**) in 25—30% yield after preparative t.l.c. separation. The treatment of the diol or its diformate (**10a**) or (**10b**) with zinc and acetic acid then produced the corresponding 19-hydroxy derivative (**11a**) or (**11b**). The 19-hydroxy group of the diformate (**11a**) was oxidised with pyridinium chlorochromate, to produce, after hydrolysis, 16α-hydroxy-19-oxotestosterone (**12b**).

Isotopic Labelling of Steroids at C-17 *and* C-19.—^{3}H Was introduced at C-17 by reducing the 17-ketone of (**9**) with NaB^3H_4 to form (**10c**) and then proceeding as for the unlabelled material to obtain (**11c**). The incorporation of ^{2}H or ^{3}H at C-19 was achieved by reduction of the aldehyde diformate (**12a**) with



Scheme 2. Synthesis of 19-functionalised derivatives of 16α-hydroxytestosterone

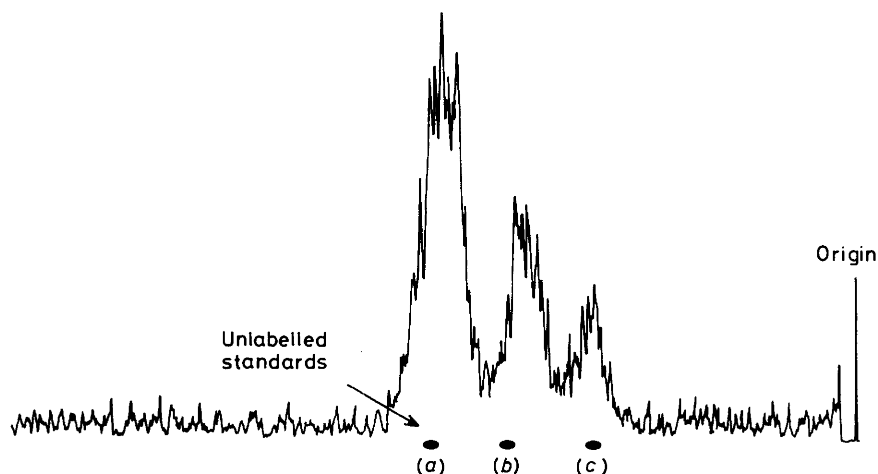


Figure. Radiochromatogram scan of t.l.c. plate showing production of oestriol (*a*) and the 19-aldehyde (**12b**), (*b*) from an incubation of the 19-alcohol (**11b**), (*c*) (0.5 μmol, 2.5 × 10^6 d.p.m.) with placental microsomes. After isolation of the bands from the plate, the amounts of radioactivity recovered were: (*a*) 7.72 × 10^5 d.p.m., (*b*) 4.87 × 10^5 d.p.m., and (*c*) 2.9 × 10^5 d.p.m. This represents an overall recovery of 55%

appropriately labelled $NaBH_4$ to form (**11d**) or (**11e**) respectively followed by deprotection to yield the triol (**11f**) or (**11g**). Our previous work[6] has shown that the major part of the label introduced by reduction of the steroidal 19-aldehyde goes into the H_{Si} position, hence the ^3H sample (**11g**) used in subsequent studies may be formulated as [80% 19*S*-^{3}H; 20% 19*R*-^{3}H]-16α,19-dihydroxytestosterone (**11g**).

The synthetic reaction sequence of the corresponding ^{2}H or ^{3}H aldehyde (**12e**) or (**12f**) contained the following precautions. In order to avoid the hydrolysis of the protecting groups, the aldehyde diformate (**12a**) was treated with a limiting quantity of [^{2}H or ^{3}H]$NaBH_4$ and the reaction mixture, without purification of the product [(**11d**) or (**11e**)], oxidised with pyridinium chlorochromate and deprotected to yield (**12e**) or (**12f**). In the deuteriated series the resulting aldehyde (**12e**) had 42 atom % excess of ^{2}H, a degree of enrichment satisfactory for the biological experiments. The material was then equilibrated with $H_2{}^{18}O/H^+$ for 17 hours at 80 °C and subjected to mass spectrometric analysis which revealed that, as expected, the carbonyl groups at C-3 and C-19 had undergone exchange with an average ^{18}O enrichment of 81 atom % excess at each position to give (**12g**) (see Experimental section). It should be noted that mass spectrometric analysis does not provide a precise assessment of ^{18}O content at each of the two exchangeable positions separately. Previous studies in the 19-oxoandrostenedione series, however, have shown that, under the above conditions, all the three atoms (at C-3, C-17, and C-19) are almost equally enriched with ^{18}O.

2. *Enzymological Studies. Study of Oestriol Biosynthesis Using the* [17α-^{3}H]-19-*Alcohol* (**11c**).—The conversion of 16α-hydroxytestosterone into oestriol was originally demonstrated by Ryan[17] and has been subsequently confirmed by other workers,[18] and by studies in our laboratory.[19] The work was extended to evaluate the role of the corresponding 19-oxygenated derivative in oestriol biosynthesis. It is, no doubt, appreciated that 17α-tritiated steroids are normally unsatisfactory for use in biosynthetic studies, since the label in this position is largely exchanged out through the redox conversion catalysed by 17β-hydroxy steroid dehydrogenases. The work of Dell'Acqua *et al.*,[18] however, suggested that steroids containing 16α-hydroxy substituents were relatively poor substrates for the 17β-hydroxy steroid dehydrogenase. In view of this information, 16α,19-dihydroxy[17α-^{3}H]testosterone (**11c**) was incubated with the microsomal preparation and when, after 1 h the mixture was worked up, 55% of the original radioactivity was extracted into the organic layer. This showed that the redox-interconversion at C-17 was more extensive than we had envisaged. Notwithstanding this, the analysis of the extract by t.l.c. showed that the majority of the radioactivity (33% of that recovered) migrated in a region corresponding to the position of authentic oestriol. The identity of the labelled material was confirmed by the elution of the band from the plate and dilution with authentic unlabelled oestriol. After formylation and crystallisation, the oestriol diformate was found to retain 91% of the original radioactivity. Another interesting feature of the radiochromatogram scan in Figure 1 is the presence of an impressive amount of radioactivity in the region corresponding to the position of 16α-hydroxy-19-oxotestosterone (**12b**). Mass spectrometric analysis of the recovered material showed the spectrum to be identical with that of authentic (**12b**). This finding not only demonstrates the formation of a 19-oxo compound (**12b**) during the conversion of the 19-hydroxy compound (**11b**) into oestriol, but also suggests that, kinetically, the intermediates of oestriol biosynthesis behave differently to those participating in the biosynthesis of oestrone. It has previously been shown[8] that in a similar incubation of 19-hydroxyandrostenedione (**2a**), the corresponding 19-oxo compound was isolated in only small amounts and its presence could only be detected during the first few minutes of the incubation. The probable reason for this difference may be traced to the finding that the participants in oestriol biosynthesis have much higher apparent K_m values compared with the corresponding derivatives in the oestrone series.[19] The higher K_m values result in weaker binding of 16α-hydroxy-19-oxygenated steroids to aromatase resulting in the dissociation of the intermediate and its accumulation in the medium. This 'exaggerated' accumulation of the 16α-hydroxy-19-oxo steroid (**12b**), lends further support to the view that, in the multistage transformation catalysed by aromatase, the 19-hydroxy and 19-oxo steroids are free intermediates and not spurious products caused by the accidental release of enzyme bound intermediates of unknown structure.[20]

The Fate of the C-19 *Hydrogen Atom(s) of* 19-*Hydroxy and* 19-*Oxo Steroids in Oestriol Biosynthesis.*—The study of the chemical details of events which occur at C-19 was based on our original approach involving the use of steroids containing ^{3}H at C-19 and determining the distribution of ^{3}H in the medium water, formaldehyde, and formic acid. For this purpose,

Table 1. The fate of C-19 hydrogen atoms of 19-hydroxy and 19-oxo steroids in oestriol biosynthesis.[a]

Compound	Incubation time (h)	Total % tritium recovered	% Tritium in sodium formate	% Tritium in water
16α,19-Dihydroxy[19-^{3}H]testosterone (**11g**) (655×10^3)	0	0.7 (4.3×10^3)		
16α,19-Dihydroxy[19-^{3}H]testosterone (**11g**) (637×10^3)	1	31 (197.4×10^3)	79 (157×10^3)	21 (41.7×10^3)
16α-Hydroxy-19-oxo[19-^{3}H]testosterone (**12f**) (643×10^3)	1	60 (385.6×10^3)	96.5 (369×10^3)	3.5 (17×10^3)
16α-Hydroxy-19-oxo[19-^{3}H]testosterone (**12f**) (652×10^3)	1	61 (398×10^3)	97 (380×10^3)	3 (11×10^3)

[a] Conversion into oestriol was demonstrated by incubating the 19-tritiated compounds (**11g**) and (**12f**) (0.5 μmol) with a microsome suspension (5 ml) containing an NADPH regenerating system for 1 h. The acid-volatile distillate was collected by freeze-drying at low pH and separated into formate and water fractions by re-freeze-drying at high pH. The formate is produced by loss of C-19 from the steroid. The measured radioactivity (d.p.m.) is shown in brackets. The location of tritium in formate was confirmed by adding unlabelled sodium formate, preparing the 4-bromophenacyl ester, and crystallising to constant specific activity. 90% Of the radioactivity remained associated with the crystals.

Table 2. The incorporation of $^{18}O_2$ or retention of 19-^{18}O in formic acid.[a]

Compound	Gas phase	Relative intensities of peaks due to benzyl esters of: $^2HCO_2H$ (m/z 137)	$^2HC^{18}OOH$ (m/z 139)	$^2HC^{18}O^{18}OH$ (m/z 141)	% Incorporation (inc.) or retention (ret.)
16α-Hydroxy-19-oxo[19-^{2}H]testosterone (**12e**)	$^{16}O_2$	99.5	0.25	0.25	
	$^{18}O_2$	22.0	71.0	7.2	71 (inc.)
	$^{18}O_2$	8.8	83.6	7.6	83.6 (inc.)
16α-Hydroxy-19-[^{18}O]oxo[3-^{18}O,19-^{2}H]testosterone (**12g**)[b]	$^{16}O_2$	23.2	72.5	4.3	89.5 (ret.)
	$^{16}O_2$	12.8	73.4	13.8	90.6 (ret.)
	$^{16}O_2$	19.0	70.4	10.6	86.9 (ret.)

[a] The compounds (**12e**) and (**12g**) (1 mg) were incubated with a microsome suspension (10 ml) in the presence of an NADPH regenerating system for 30 min at 37 °C under $^{16}O_2$ or $^{18}O_2$ as shown. After addition of orthophosphoric acid (0.8M; 2 ml), the acid-volatile distillate was collected by freeze-drying, converted into the benzyl derivative, and analysed by g.l.c.-m.s. The percentage incorporation or retention of ^{18}O is obtained from the relative intensity of the peak due to $^2HC^{18}OO$ Bz (m/z 139). [b] 42% Excess ^{2}H, 81% average excess ^{18}O.

[19-^{3}H]-19-oxo steroid (**12f**) was incubated aerobically with the microsomal fraction, in the presence of an NADPH regenerating system. After 1 h the incubation medium was freeze-dried and the distillate further fractionated for the determination of radioactivity in ^3H_2O and ^{3}HCOOH. Table 1 shows that in such experiments 60% of the original radioactivity was released into the medium and, of this, at least 96% was found to be associated with ^{3}HCOOH when the latter was analysed as the 4-bromophenacyl derivative. Similar experiments were performed with the [19-^{3}H]-19-hydroxy compound (**11g**) and Table 1 shows that in this case about 30% of the original C-19 radioactivity was released into the medium and that its distribution between medium ^3H_2O and ^{3}HCOOH was in the ratio 21:79. From earlier work[6] it may be estimated that in the chemical introduction of the label at C-19 of (**11g**), about 80% of the ^{3}H was in the H_{Si} position and 20% at H_{Re}. The predominant retention of ^{3}H in HCOOH means that H_{Si} was not disturbed in the overall reaction and, hence, H_{Re} must have been removed in the oxidation attending the conversion (**2c**) ⟶ (**3c**). This result has been obtained before for the corresponding conversion (**2a**) ⟶ (**3a**) in the biosynthesis of oestrone.[6]

^{18}O *Content of* C-19 *Released as Formate*.—The status of oxygen in the conversion of the aldehyde (**12b**) into oestriol was studied using two incubation protocols in parallel. In one, [19-^{2}H, 19-^{18}O]aldehyde (**12g**) (1 mg) was incubated with placental microsomes for 30 min, as described in Table 2, under O_2, whilst in the other, [19-^{2}H]aldehyde (**12f**) (1 mg) was similarly treated, but under $^{18}O_2$. In both cases about 40—60 μg of sodium formate was produced. The samples were converted into benzyl formate and analysed by g.l.c.-m.s.[8] Table 2 shows that the values for retention of the original C-19 oxygen of the aldehyde, as indicated by the formate produced and the incorporation of one oxygen atom from $^{18}O_2$ in formate, are more than 80% of that predicted by the sequence shown in Scheme 1 (see the status of oxygen atoms marked by ◒ and ●).

Discussion

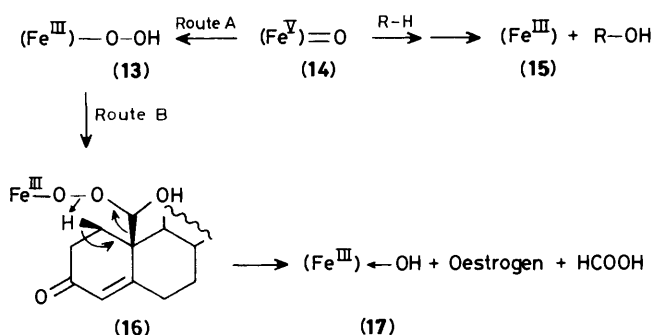
Cumulatively, these results are consistent with the notion that 16α-hydroxytestosterone (**1c**) (Scheme 1) is converted into oestriol (**4c**), *via* its 19-hydroxy and 19-oxo derivatives and that the overall molecular changes occurring during the transformation are identical with those already established for the conversion of androstenedione (**1a**) into oestrone (**4a**).* The present work validates the proposal that in the conversion (**3c**) ⟶ (**4c**), one atom of oxygen from O_2 is incorporated into

* In view of the apparent mechanistic similarity between the aromatisation of 16α-hydroxy and 16-deoxy androgens, it is quite reasonable to envisage that both processes could be carried out by a single enzyme, which can accommodate either the presence or absence of a 16α-hydroxy group. Our own studies,[19] are consistent with the view, though the involvement of two different aromatases for the two series has been considered in the literature [Y. Osawa, B. Tochigi, T. Higashiyama, C. Yarborough, T. Nakamura, and T. Yamamoto, *Cancer Res.* (*Suppl.*), 1982, **42**, 3299s]. A detailed discussion of this question is beyond the scope of this paper.

the expelled formate, and provides further evidence that this incorporation is the key fact to be explained when considering the mechanism of the C-10–C-19 cleavage step.

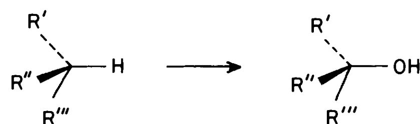
In an alternative mechanistic proposal, the third mole of O_2 is used to produce a 2β-hydroxy-19-oxo steroid which fragments non-enzymically[1c] to produce the aromatic ring A of oestrogens. For this mechanism to be consistent with the ^{18}O labelling experiments discussed above, the oxygen atom of the 2β-hydroxy group must be incorporated into the formate released. Recently, Caspi *et al.*[21] studied the aromatisation of 2β-[^{18}O]hydroxy-19-oxoandrost-4-ene-3,17-dione but failed to detect any ^{18}O in the formate produced. This suggests that the 2β-hydroxy compound is not an obligatory intermediate in oestrogen biosynthesis.

The mechanism originally proposed[8] by us and further elaborated[12] in Scheme 3 thus remains a strong contender. According to this proposal, the third mole of oxygen is involved in the formation of an enzyme-peroxide species (**13**) that attacks the C-19 aldehyde causing elimination as shown in Scheme 3 (Route B). An Fe^{III}-peroxide species is a postulated intermediate in the 'normal' catalytic cycle of cytochrome P-450 (Scheme 3, Route A). Our proposed mechanism envisaged that the reaction of the peroxide with the aldehyde is faster than the formation of the enzyme-mono-oxygen species (**14**) which would otherwise follow in preparation for a hydroxylation process. Since this proposal was made, however, there have been interesting developments in the field of P-450 dependent hydroxylation reactions and these suggest that a further definition of the proposal of Scheme 3 may involve the participation of free-radical intermediates rather than the concerted process shown in Scheme 3 (Route B). With this realisation, seemingly unrelated processes, *e.g.* hydroxylation reactions, oxidation of alcohols to carbonyl compounds, and a variety of C–C bond cleavage reactions, catalysed by aromatase and other P-450 cytochromes, may be viewed as variations on a common mechanistic theme. Here we consider this hypothesis along with the relevant observations from the existing literature, which have assisted in the formulation of these views.

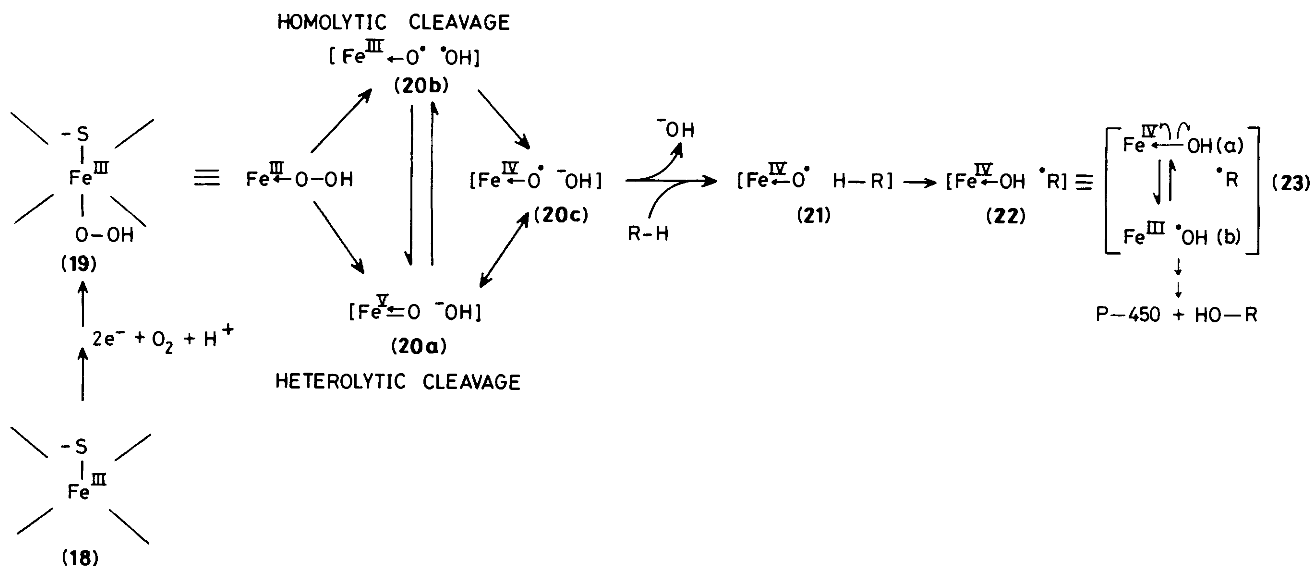


Scheme 3. Alternative pathways showing the dual role of a P-450-peroxide species (13) in hydroxylation and C–C bond cleavage steps

The Hypothesis.—The classical work of Gunsalus on the camphor hydroxylase system, defined the chemistry of the stepwise addition of two electrons and one mole of O_2 to P-450, producing an adduct that was formulated as the iron-peroxide species (**19**) (Scheme 4).[22] The generality of this process has subsequently been established for several other hydroxylases. The evidence for the fact that a P-450 mono-oxygen species (**20**) is involved in the final hydroxylation process, RH ⟶ ROH, came from a series of indirect experiments where it was shown that in the presence of certain artificial oxidants ($NaIO_4$,[23a] iodosobenzene,[23b] organic peroxides[23c]), P-450 catalysed hydroxylation reactions occurred, without requiring NADPH and O_2. Chemical considerations dictate that, in these cases, the only oxygenated species that can form is the P-450 mono-oxygen complex (**20**); this must be involved in artificial hydroxylation and, hence by implication, in the physiological process. Three forms of the P-450 mono-oxygen species are represented in structures (**20a**)—(**20c**) of Scheme 4. The question as to which of these forms participates in the final hydroxylation process and the detailed chemistry of the cleavage of the R–H bond remained unclear for nearly two decades.[23d,24a] The underlying cause of this was the repeated demonstration that a variety of aliphatic hydroxylation reactions occurred with overall retention of stereochemistry, *i.e.*,



Consequently, it became an obligatory requirement that any accepted mechanism of hydroxylation must also explain the



Scheme 4. A free radical mechanism for P-450 (18) dependent hydroxylation reactions

retention of stereochemistry, for which no precedent was known. Attention, however, was drawn to the fact that certain reactive carbenes (CH_2:), could insert into C–H bonds to yield products with retention of stereochemistry.[23d,24a,b] It was hence deduced that the complex (**20a**) may generate 'oxene' (an oxygen counterpart of carbene) that may insert into a C–H bond, producing a hydroxylated product, with definite retention of stereochemistry. Two recent studies, however, have shown that some of the earlier conclusions were erroneous and in several cases the hydroxylation reactions had occurred with racemisation, thus highlighting that the cleavage of a C–H bond and its conversion into C–OH, must in fact be a stepwise process involving intermediates.[25]

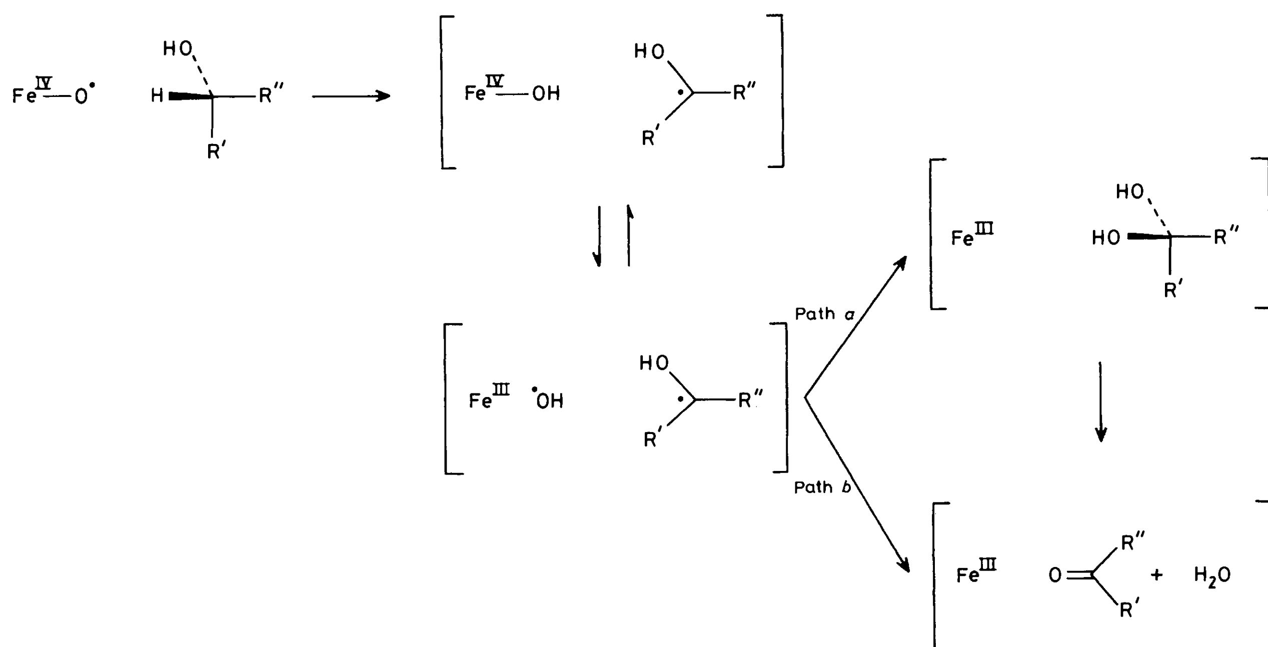
The cleavage of the C–H bond can occur by one of three routes involving a carbanion, carbonium ion, or radical. The last process is most likely on chemical grounds, especially when the hydrogen to be abstracted is bonded to a non-activated position, as is the case in most P-450 dependent aliphatic hydroxylations. Groves *et al.*[26] first suggested that a ferroxy radical, represented by the canonical form (**20c**) may be a suitable candidate for hydrogen abstraction, leading to the formation of a carbon radical which is neutralised by association with the hydroxy radical. This proposal has subsequently been supported by others.[24a] The driving force for the formation of the hydroxy radical from (**23a**) must be the energetic advantage accruing from the conversion of Fe^{IV} into Fe^{III}.

Mechanism of the P-450 *Dependent Oxidation of Alcohols into Carbonyl Compounds*.—The stage was now set to extrapolate the proposal to other generic reactions catalysed by aromatase. Influenced by the prevailing belief that the activated oxygen species generated in P-450 linked reactions can only insert into C–H bonds through a concerted process, we had suggested that during oestrogen biosynthesis the conversion of the 19-hydroxy compound (**2a**—**c**) into the 19-aldehyde (**3a**—**c**) occurs through the participation of a *gem*-diol. The constraint of a concerted process having been removed, the conversion $-CH_2OH \longrightarrow -CH{=}O$ may be rationalised by either of the two processes shown in Scheme 5. A clear choice between these two alternatives (path *a* and path *b*, Scheme 5) is not yet possible, but our previous experiments[8] show that in the conversion (**2a**) $\longrightarrow$ (**3a**), the hydroxy oxygen atom of (**2a**) is retained and this would be consistent with the more direct path *b* involving the usual hydrogen abstraction followed by disproportionation.*

Mechanism of the P-450 *Dependent Cleavage of* C–C.—In the light of the theory developed above (which views the hydroxylation and oxidation reactions as radical processes) the mechanism of the C-10–C-19 bond fission in oestrogen biosynthesis may also be described in terms of a radical process for which a number of closely related alternatives exist. For example the $Fe^{III} \longleftarrow OOH$ species produced as an intermediate in P-450 linked reactions (*cf.*, Scheme 4) will be expected to possess strong nucleophilic properties for addition to the C-19 carbonyl group producing the intermediate (**24**) (Scheme 6, Panel A). The homolytic cleavage of the peroxy bond of the latter (**24**) will then furnish the alkoxy radical of the type (**25**). There are numerous precedents[27] to suggest that a favoured course for the decomposition of such an alkoxy radical (**25**) is fragmentation to produce formate and a tertiary carbon radical which is quenched by a disproportionation process creating the 1,2-ene system of oestrogen. For illustration purposes, the keto-enol isomerisation step in the sequence of Panel A is shown as the terminal event, though the possibility that the isomerisation may in fact precede the bond fission cannot be ruled out.

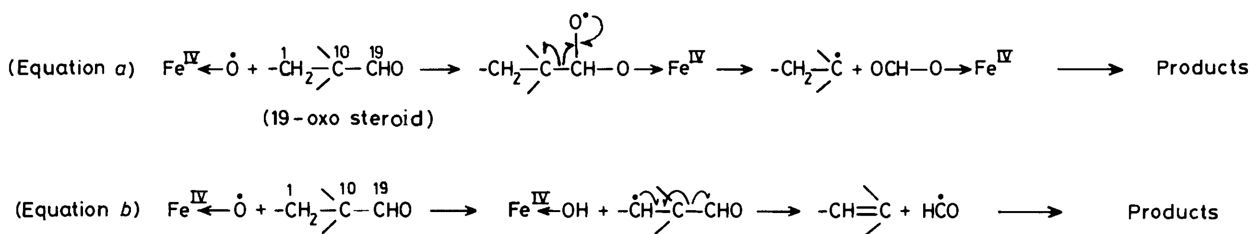
An alternative sequence to that in Panel A involves the ferroxy radical ($Fe^{IV} \longleftarrow \dot{O}$) which may promote the cleavage of the C-10–C-19 bond either *via* a hemiacetal radical (Equation *a*, Scheme 7) or by hydrogen abstraction from C-1 (Equation *b*). The merit of the proposals in Equations *a* and *b* (Scheme 7) is that, unlike the mechanism of Panel A, the same $Fe^{IV} \longleftarrow \dot{O}$ species used in the C–C bond cleavage step is the same as that in the preceding hydroxylation and oxidation reactions. The main argument against the sequence of Equation *a* lies in justifying why the initial reaction of $Fe^{IV} \longleftarrow \dot{O}$ with the CHO

* The operation of pathway *a* for the oxidation of an alcohol into a carbonyl compound has recently been indicated using an artificial substrate (D. D. Beusen, H. L. Correll, and D. F. Covey, *Biochemistry*, 1987, **26**, 7833).



Scheme 5. Mechanism of P-450 dependent oxidation of alcohols into carbonyl compounds

Scheme 6. Mechanism of P-450 dependent cleavage of C–C bonds. Panel A, aromatase catalysed process; Panel B, the removal of the 14α-methyl group in sterol biosynthesis; Panel C, formation of Δ^{16}-androgen from pregnenolone, and Panel D, formation of androgens from 17α-hydroxypregnenolone. In Panels A, B, and D only the final bond-cleavage steps are considered, although in each case the same enzyme catalyses the preceding oxidation step(s) not shown here



Scheme 7.

group results in adduct formation and not hydrogen abstraction causing further oxidation of C-19 to a carboxylic acid. The weakness in the sequence of Equation *b* lies in the creation of a reactive species ($H\dot{C}O$) that is too small to be adequately held at the active-site and is therefore likely to undergo random decomposition rather than be directed in the desired course of reaction. The choice between the two mechanisms (Panel A, Scheme 6 *vs.* Scheme 7) must await experiments in which individual stages in oestrogen biosynthesis are studied using an artificially generated $Fe^{IV} \longleftarrow \dot{O}$ species.

The mechanism proposed for the aromatase catalysed reaction in Scheme 6, Panel A can, without modification, be extended to several other lyase reactions involved in steroidogenesis. We have already provided strong evidence[28] to show that the enzymes involved in the removal of the 14α-methyl group, in the biosynthesis of all classes of sterols, are related to aromatase and the mechanism of the crucial C-14–C-32 bond cleavage step therein, may now be elaborated as in Scheme 6, Panel B. The cleavage of the side-chain in androgen biosynthesis[29] and the formation of Δ^{16}-androgens[30] are the other

examples of the same generic reaction and their mechanisms are presented in Scheme 6, Panels D and C, respectively. The cholesterol side-chain cleavage process has been explained by a minor variation of this type of mechanism (for example, see an earlier proposal).[31]

Stereochemical Control in P-450 *Dependent Reactions*.—If the above thesis is accepted, then the retention of stereochemistry generally observed in hydroxylation reactions and the elimination of a hydrogen atom *cis* to the scissile C–C bond, that has been established for the conversion of Panels A and B (Scheme 6) and predictable for that of Panel C, are not consequences of the reaction mechanism itself. They are, however, due to the constraints on the mobility of the substrate within the Michaelis complex. It makes sense to assume that enzyme systems operating through the intermediacy of particularly reactive species such as free radicals have evolved to allow a minimum motion of bonds during catalysis, in order to ensure that these species do not participate in random reactions and damage the nature of the active site.

Experimental

Chemicals were generally obtained from B.D.H. Ltd., Poole, Dorset, U.K. except for the following: 3β-hydroxyandrost-5-en-17-one (DIA) acetate, NAD, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (Sigma Chemical Co., Poole, Dorset, U.K.), 1,1′-carbonyldi-imidazole (CDI) and NaB^2H_4 (Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.), $H_2{}^{18}O$ (99 atom % excess), $^{16}O_2$ and $^{18}O_2$ (99 atom % excess) (Prochem. B.O.C. Ltd., London, U.K.) and NaB^3H_4 (The Radiochemical Centre, Amersham, Bucks, U.K.). Solvents were purified by redistillation. Ether refers to diethyl ether.

M.p.s were measured on an electrothermal melting-point apparatus and are uncorrected. I.r. spectra were recorded on a Unicam S.P. 1000 I.R. Spectrometer and n.m.r. spectra were recorded either on a Perkin-Elmer R12B (60 MHz) spectrometer or by Southampton University Chemistry Department on a Bruker WM 360 instrument. Mass spectra were recorded on an A.E.I. M.S.30 instrument.

Measurement of radioactivity was carried out in Butyl PBD [2-biphenyl-4-yl-5-(*p*-t-butylphenyl)-1,3,4-oxadiazole] (Ciba, Basle, Switzerland) in toluene (10 ml, 8 g l^{-1}) or, for aqueous samples, in 10 ml of water-miscible scintillant in a Phillips PW4700 Liquid Scintillation Counter, programmed for automatic quench correction. Values recorded in d.p.m. have been corrected to 100% efficiency.

Analytical and preparative t.l.c. plates were prepared from silica gel GF_{254} and PF_{254}, respectively (Merck, Darmstadt, F.R.G.) according to the manufacturer's instructions. Radiochromatogram scanning was performed with a Berthold LB2723 scanner.

5α-*Bromo*-3β-*hydroxy*-6β,19-*epoxyandrostan*-17-*one* (**6**).—This was prepared from DIA (**5**) by the method of Akhtar *et al.*[13]

5α,16α-*Dibromo*-3β-*hydroxy*-6β,19-*epoxyandrostan*-17-*one* (**7**).—This was prepared by a modification of the 16-bromination technique of Glazier.[14] Compound (**6**) (1 g) and copper(II) bromide (1 g) in dry ethanol (100 ml) were refluxed for 24 h. The precipitate of copper(I) bromide was filtered off, and the solution was poured into distilled water (200 ml) and extracted with methylene dichloride (3 × 50 ml). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure to yield a brown oil. After crystallisation from methanol, white needles (0.8 g) were obtained, m.p. 206—208 °C; $[\alpha]_D^{23}$ +20.25° (*c* 2.0, $CHCl_3$); $\nu_{max.}$(Nujol) 3 500 and 1 734 cm^{-1}; $\delta_H(CDCl_3)$ 0.92 (3 H, s, 18-Me), 3.75 (2 H, m, 19-CH_2), 4.0 (1 H, s, 6-H), *ca.* 4 (1 H, br s, 3-H), and 4.45 (1 H, t, 16-H) (Found: C, 49.2; H, 5.7. $C_{19}H_{26}Br_2O_3$ requires C, 49.37; H, 5.67%).

5α,16α-*Dibromo*-6β,19-*epoxyandrostane*-3,17-*dione* (**8**).—This was prepared from compound (**7**), using Jones reagent.[15] Crystallisation from methylene dichloride–light petroleum afforded white crystals (80%). Only spectroscopic analysis was performed on this compound since it decomposes, with loss of HBr, even at −20 °C; $\nu_{max.}$(Nujol) 1 722 cm^{-1}; $\delta_H(CDCl_3)$ 0.94 (3 H, s, 18-Me), 4.0 (3 H, m, 19-CH_2, 6-H), and 4.4 (1 H, s, 16-H).

16α-*Hydroxy*-6β,19-*epoxyandrost*-4-*ene*-3,17-*dione* (**9**).—This was prepared from compound (**8**) by adapting the method of Numazawa and Nagaoka,[16] with the difference that 2 equiv. of potassium carbonate were used instead of 1. The product was crystallised from methanol–ether to yield white crystals (60—70%). T.l.c. analysis indicated contamination with a small amount of the 16-bromo compound so, for analytical purposes, a sample was purified by preparative t.l.c. using methylene dichloride–methanol (96:6). After further crystallisation, the following data were obtained: m.p. 200—203 °C; $[\alpha]_D^{25}$ −24.2° (*c* 2.5, $CHCl_3$); $\nu_{max.}$(Nujol) 3 430, 1 737, and 1 645 cm^{-1}; $\delta_H(CDCl_3)$ 1.0 (3 H, s, 18-Me), 3.78 (2 H, m, 19-CH_2), 4.3 (1 H, s, 16-H), 4.63 (1 H, d, 6-H), and 5.72 (1 H, s, 4-H) (Found: C, 72.1; H, 7.8. $C_{19}H_{24}O_4$ requires C, 72.12; H, 7.65%).

16α,17β-*Dihydroxy*-6β,19-*epoxyandrost*-4-*en*-3-*one* (**10a**).—Compound (**9**) (100 mg) was treated with sodium borohydride (14 mg) in methanol (10 ml) for 15 min at −21 °C. The reaction was terminated by addition of acetic acid (0.2 ml), the solvents were evaporated under reduced pressure, and the residue was dissolved in ethyl acetate, washed with aqueous sodium hydrogen carbonate, and dried (Na_2SO_4). The solution was evaporated to 1 ml under reduced pressure and applied to two 20 × 20 cm preparative t.l.c. plates. After development in methylene dichloride–methanol (12:88), the u.v.-absorbing band at R_F 0.2 was scraped off and eluted with ethyl acetate–methanol (1:1). The product was crystallised from methylene dichloride–light petroleum to yield white needles (25—30 mg), m.p. 189.5—192 °C; $[\alpha]_D^{23}$ −149.4 (*c* 2.0, MeOH); $\nu_{max.}$(Nujol) 3 400 and 1 665 cm^{-1}; δ_H(CD_3OD, 360 MHz) 0.86 (3 H, s, 18-Me), 3.86 (2 H, m, 19-CH_2), *ca.* 3.4 (17 H superimposed on CD_3OH signal), 4.0 (1 H, t, 16-H), 5.84 (1 H, s, 4-H), and *ca.* 4.7 (6-H superimposed on H_2O signal). These δ values are almost identical (apart from the resonance due to the 6,19-epoxide) with those of 16α-hydroxytestosterone,[32] indicating that the stereochemistry was the required 16α,17β. Mass spectral analysis gave M^+, 318.1873. $C_{19}H_{26}O_4$ requires M, 318.1830.

16α,17β,19-*Trihydroxyandrost*-4-*en*-3-*one* (**11b**).—Compound (**10a**) (100 mg) in acetic acid–water (20:1; 4 ml) was treated for 8 min with zinc dust (0.7 g) whilst being stirred rapidly under reflux. Concentrated hydrochloric acid (20 μl) was added and the reaction was continued for a further 6 min. The mixture was gravity filtered into saturated aqueous sodium hydrogen carbonate (150 ml) and the product extracted with methylene dichloride (3 × 20 ml); the organic extracts were pooled, washed with aqueous sodium hydrogen carbonate, dried (Na_2SO_4), and the solvents evaporated under reduced pressure. The resulting oil was crystallised from methanol–ether to yield powdery white crystals (43 mg), m.p. 235—238 °C; $\nu_{max.}$(Nujol) 3 340, 1 650, and 1 605 cm^{-1}; δ_H(CD_3OD, 360 MHz) 0.78 (3 H, s, 18-Me), 3.35 (d, 17-H, superimposed on CD_3OH peak), 3.85 (1 H, d, 16-H), 4.0 (2 H, d, 19-CH_2), and 5.88 (1 H, s, 4-H) (M^+, 320.1985. $C_{19}H_{28}O_4$ requires M, 320.1987).

Similar results were obtained when the 6β,19-epoxy ring opening was carried out using the method of Akhtar *et al.*[8]

3-*Oxo*-6β,19-*epoxyandrost*-4-*ene*-16α,17β-*diol* *Diformate* (**10b**).—Compound (**10a**) (93 mg) was added to a mixture of formic acid (0.132 ml) and 1,1′-carbonyldi-imidazole (0.568 g) in dry methylene dichloride (5 ml) and the mixture left overnight. The solution was diluted to 20 ml, washed with dilute hydrochloric acid and aqueous sodium hydrogen carbonate, dried (Na_2SO_4), and the solvent evaporated under reduced pressure. The resulting oil was crystallised from methanol–ether, to yield white needles (90 mg), m.p. 210.5—213 °C; $[\alpha]_D^{23}$ −178.49° (*c* 1.6, $CHCl_3$); $\delta_H(CDCl_3)$ 0.92 (3 H, s, 18-Me), 3.78 (2 H, m, 19-CH_2), 4.63 (1 H, d, 6-H), 5.1 (2 H, m, 16,17-H), 5.75 (1 H, s, 4-H) and 7.91 and 8.02 (2 H, 2 × s, 16-, 17-formate) (M^+, 374.1767. $C_{21}H_{26}O_6$ requires *M*, 374.1730).

3-*Oxoandrost*-4-*ene*-16α,17β,19-*triol* 16α,17β-*Diformate* (**11a**).—This was prepared from compound (**10b**) by the method used to prepare (**11b**). Compound (**10a**) (200 mg) yielded white crystals (125 mg), m.p. 196—198.5 °C; $[\alpha]_D^{23}$ −4.55° (*c* 1.0, $CHCl_3$); $\delta_H(CDCl_3)$ 0.85 (3 H, s, 18-Me), 3.9 (2 H, s, 19-H_2), 5.1 (2 H, m, 16,17-H), 5.85 (1 H, s, 4-H), and 7.89, 7.98 (2 H, 2 × s, formates) (M^+, 376.1856. $C_{21}H_{28}O_6$ requires *M*, 376.1890).

3,19-*Oxoandrost*-4-*ene*-16α,17β-*diol* *Diformate* (**12a**).—Compound (**11a**) (250 mg) was treated with pyridinium chlorochromate (187 mg) in dry methylene dichloride (5 ml) for 2 h. Ether (15 ml) was added and the mixture filtered through a 1 × 1 cm silica gel column to remove the reagent. After evaporation of the solvents under reduced pressure, the product was crystallised from methanol to yield white crystals (180 mg), m.p. 146—148 °C; $[\alpha]_D^{23}$ +78.77° (*c* 1.0, $CHCl_3$); $\delta_H(CDCl_3)$ 0.83 (3 H, s, 18-Me), 5.05 (2 H, m, 16,17-H), 5.85 (1 H, s, 4-H), 7.85, 7.94 (2 H, 2 × s, formates), and 9.75 (1 H, s, 19-H) (M^+, 374.1712. $C_{21}H_{26}O_6$ requires *M*, 374.1730).

16α,17β-*Dihydroxyandrost*-4-*ene*-3,19-*dione* (**12b**).—Compound (**12a**) (40 mg) was treated with a 5% solution of sodium hydrogen carbonate in 5% aqueous methanol (3 ml) for 2 h. The mixture was acidified with dilute hydrochloric acid, partitioned between ethyl acetate and water, and the combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The resulting oil was crystallised from methanol–ether to yield white crystals (16 mg), m.p. 191—194 °C; $\delta_H(CD_3OD$, 360 MHz) 0.78 (3 H, s, 18-Me), 3.35 (1 H, d, 17-H), 3.5 (1 H, m, 16-H), 5.94 (1 H, s, 4-H), and 9.95 (1 H, s, 19-H) (M^+, 318.1851. $C_{19}H_{26}O_4$ requires *M*, 318.1830).

Synthesis of Isotopically Labelled Compounds.—Except where otherwise stated, labelled steroids were interconverted by scaled down versions of the synthesis of the unlabelled equivalents. Products were purified at each stage using preparative t.l.c. with authentic unlabelled material as a reference. Yields were assessed by measuring the u.v. absorbance of the conjugated ketone system, common to all the compounds. Except where yields were large enough for crystallisation, compounds were stored in methanol solution at −20 °C. Before biological experiments purity was checked by t.l.c. and the compounds repurified, if necessary.

16α,17β,19-*Trihydroxy*[17α-^{3}H]*androst*-4-*en*-3-*one* (**11c**).—The 17-ketone (**9**) (50 mg) was reduced to the diol (**10c**) as for the unlabelled material, except that *ca.* 0.1 equiv. of NaB^3H_4 (452 mCi $mmol^{-1}$) was added and the reaction was allowed to proceed for 5 min before the unlabelled borohydride was added. The epoxide was opened and after purification by t.l.c. (methylene dichloride–methanol, 80:20), compound (**11c**) (5 mg, 9.7 × 10^7 d.p.m. mg^{-1}) was obtained.

Synthesis of the 19-*Tritiated* 19-*Alcohol and* 19-*Aldehyde* (**11g**) *and* (**12f**).—The 3,19-dioxo diformate (**12a**) (30 mg) was reduced with NaB^3H_4 (452 mCi $mmol^{-1}$) according to the method used in the androstenedione series. After purification (t.l.c., methylene dichloride–methanol, 95:5) half the product was deprotected and purified by t.l.c. (methylene dichloride–methanol 80:20) to yield (**11g**) (3 mg, 5 × 10^8 d.p.m. mg^{-1}). The remainder was oxidised to (**12d**), purified, and deprotected to yield, after purification by t.l.c. (methylene dichloride–methanol, 80:20) (**12f**) (3 mg, 2 × 10^8 d.p.m. mg^{-1}).

Synthesis of 19-*Deuteriated* 19-*Alcohol and* 19-*Aldehyde* (**11f**) *and* (**12e**).—This synthesis was analogous to that for the 19-tritiated compounds (*vide supra*). Compound (**12a**) (25 mg) was reduced and deprotected to yield, after two crystallisations (**11f**) (8 mg). The product was pure and behaved identically by t.l.c. to the unlabelled material. The mass spectrum of the product showed the same fragmentation pattern as the unlabelled material and no unlabelled impurity could be detected.

In order to prepare the 19-aldehyde (**12e**), the 19-aldehyde diformate (**12a**) (50 mg) was reduced using half the normal quantity of NaB^2H_4. Normally there is significant methanolysis of the formyl groups in the presence of the basic borohydride, thus it was decided to maximise yield at the cost of decreased incorporation of label, in order to obtain sufficient compound. After re-oxidation of the crude product and deprotection, followed by two recrystallisations, the product (22 mg) was obtained. T.l.c. analysis showed a single spot corresponding to authentic unlabelled material. The mass spectrum of the product showed the same fragmentation pattern as the unlabelled material and examination of the molecular ion peaks due to labelled and unlabelled compond indicated a deuterium content of 42% excess at C-19. It should be possible to increase the incorporation of label to 60—80% at the cost of a much lower yield.

[16α,17β-*Dihydroxy*[19-^{2}H,3,19-$^{18}O_2$]*androst*-4-*ene*-3,19-*dione* (**12g**).—Compound (**12e**) (15 mg) was sealed into a glass tube with dry isopropyl alcohol (2 ml), containing 0.5 × 10^{-2}M HCl and $H_2{}^{18}O$ (99 atom % excess, 25 μl) and the tube was heated in refluxing benzene for 17 h. After addition of water (5 ml), the product was extracted with ethyl acetate, dried briefly (Na_2SO_4), and recrystallised twice from methanol–ether to yield (**12g**) (4.5 mg). T.l.c. showed a single spot corresponding to the authentic unlabelled material.

This compound should contain ^{18}O at C-3 and C-19 and there were, as expected, six molecular ion peaks in the mass spectrum of the product, due to steroid with 0, 1, and 2 atoms of ^{18}O (*m*/*z*: 318, 320, 322) and with 0, 1, and 2 atoms of ^{18}O and 1 atom of ^{2}H (*m*/*z*: 319, 321, 323). The ratios of intensities of the peaks at 318/319, 320/321, and 322/323 were the same at 58:42, indicating that the deuterium content was unchanged. The ratios of the peaks at 318/320/322 and 319/321/323 were 5.04:28.3:66.7 and 6.3:29.2:64.4, respectively, giving an average ^{18}O content of 81%, from the formula % excess = $10\sqrt{(M+4)\%}$ where $(M + 4)\%$ is the percentage of molecules containing two atoms of ^{18}O.

Preparation of Placental Microsomes.—The microsomal fraction was prepared as described previously.[33]

Incubations with 19-*Tritiated* 19-*Functionalised Steroids.*—Thawed microsomal suspension (5 ml) was placed in a 50 ml round-bottom flask and a NADPH generating system was developed using the following: NADP (1mM), glucose-6-phosphate (6mM) and glucose-6-phosphate dehydrogenase (0.5 units ml^{-1}. The steroid (0.5 μmol) plus radioactive tracer (see

Table 1) was added in methanol solution (0.1 ml) and the mixture was incubated in air at 37 °C in a shaking water bath for 1 h. The mixture was acidified with 0.8M orthophosphoric acid (1 ml) and freeze-dried; the acid-volatile distillate was collected in a cold-trap. A 1 ml aliquot of the acid-volatile distillate was removed for scintillation counting, to determine the total yield of tritium. The remainder was raised to pH 10 with sodium hydroxide solution (0.5M) and unlabelled sodium formate (50 mg) was added. The mixture was again freeze-dried; formate being involatile, only the water distills into the trap. Aliquots of water and formate were removed for scintillation counting, to determine the distribution of tritium between water and formate fractions.

In order to confirm the identity of the sodium formate, the solid was dissolved in wet ethanol (5 ml) and 4-bromophenacyl bromide (100 mg) was added. The mixture was heated under reflux for 40 min and the resulting 4-bromophenacyl formate was precipitated with water, filtered, and crystallised to constant specific activity. In all cases, at least 90% of the radioactivity was retained in the crystals.

Incubations with 16α,17β,19-*Trihydroxy*[17α-^{3}H]*androst*-4-*en*-3-*one* (**11c**).—Compound (**11c**) (0.5 μmol, 2.5 × 10^6 d.p.m.) was incubated as in the previous section. After 1 h the microsomes were diluted with distilled water (50 ml) and extracted with ethyl acetate–ether (4:1, 3 × 75 ml). The organic extracts were combined, dried (Na_2SO_4), and reduced to 0.5 ml under reduced pressure. The solution was applied to a 10 × 20 cm preparative t.l.c. plate along with standards of oestriol (**11b**) and (**12b**). After running the plate twice in methylene dichloride–acetone (1:1) the bands were visualised with u.v. light, molybdophosphoric acid spray, and by radiochromatogram scanning. The bands of radioactivity were scraped off the plate and eluted. After removing samples for scintillation counting, the oestriol was mixed with authentic unlabelled material, converted into the formate, and crystallised to constant specific activity, while the identity of the 19-aldehyde was confirmed by mass spectroscopy.

Experiments with ^{18}O *Substrates and* $^{18}O_2$.—These incubations and the isolation, derivatisation, and analysis of the formic acid produced were carried out as described previously.[8]

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