The Status of Oxygen Atoms in the Removal of C-19 in Oestrogen Biosynthesis

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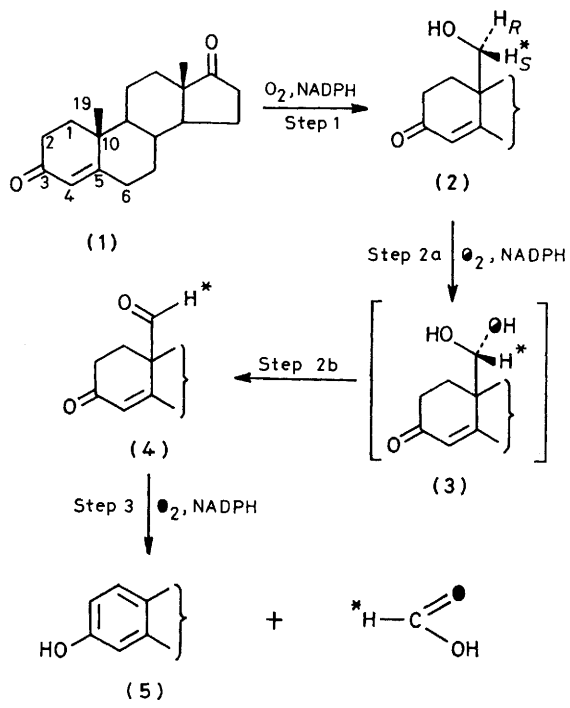
Summary Three O_2-dependent reactions are involved in the removal of C-19 as formate, in oestrogen biosynthesis; it is shown that the oxygen atoms introduced in steps 1 and 3 of the process are the ones which are found in the biosynthetic formate.

THE biosynthesis of oestrone (**5**) from androstenedione (**1**) in human placenta comprises three steps, in which oxygen and NADPH are required, and proceeds through at least two 19-oxygenated intermediates, 19-hydroxy- (**2**) and 19-oxo-androstenedione (**4**).[1] Previous studies on the nature of biochemical events occurring at C-19 during oestrogen biosynthesis, using tritiated C-19 substrates,[2] have now been extended to include two types of experiment using ^{18}O. The deuteriated steroids (**2**, H* = D), (**4**, H* = D), and non-deuteriated (**1**) were biologically converted into oestrone (**5**) under $^{18}O_2$, and ^{18}O-labelled steroids (**2**, H* = D, O = ^{18}O) and (**4**, H* = D, O = ^{18}O) were similarly converted under $^{16}O_2$.

The status of the oxygen atoms during each step of the transformation was determined from the analysis, by g.l.c.–mass spectrometry, of the deuteriated formate† (measured as benzyl formate[3]) released from C-19. The biosynthetic experiments involved the incubation of substrates [(**1**), (**2**), or (**4**); 500 μg] with placental microsomes[4] (equivalent to 250 mg of protein) in the presence of an NADPH regenerating system, and under an atmosphere of $^{16}O_2$ or $^{18}O_2$ as appropriate, in a final volume of 5 ml at 37 °C, for a period of 20 min. The results are in the Table, and the conclusions from the biosynthetic experiments, summarized below, are best understood by considering step 3 of Scheme 1 first, then step 2, and finally step 1.

(i) In the conversion of the deuterio-aldehyde (**4**, H* = D) into oestrone (**5**) under $^{18}O_2$ at least 90% of the deuterio-formate molecules released from C-19 contained one atom of ^{18}O. This result shows a substantially higher ^{18}O incorporation over the data reported previously,[5] in which the protium analogue of the aldehyde (**4**) was generated *in situ*. Our present findings support the assertion then made that a novel oxidative mechanism may operate for the cleavage of the C(10)–C(19) bond in oestrogen biosynthesis.



SCHEME 1.

When the [19-^{18}O]aldehyde (**4**, H* = D; O = ^{18}O) was converted into oestrone (**5**), under $^{16}O_2$, the aldehydic oxygen was retained to the extent of 82% in the released formate. This experiment proves that the C-19 carbonyl group of compound (**4**) neither participates in Schiff-base formation nor is transferred to a carrier, such as tetrahydrofolate, during the cleavage reaction.

(ii) The hydroxy-oxygen atom of (**2**) was transferred to formate to the extent of 90%, thus implying that in the conversion of the alcohol (**2**) into the aldehyde (**4**) the original oxygen of the former is retained. In the opposite

TABLE. The ^{18}O content of formate released from C-19 during oestrone biosynthesis.

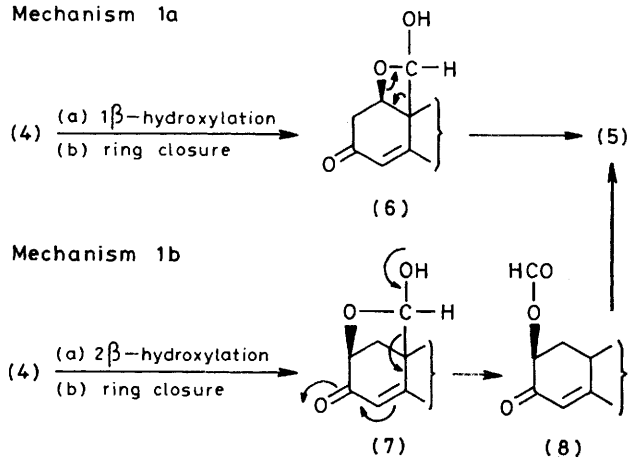
Substrate	%^{18}O atom excess in substrate	Gas phase	Relative intensities of peaks due to $^2HCO_2H$ *m/z* 137	$^2HC^{18}OOH$ *m/z* 139	$^2HC^{18}O^{18}OH$ *m/z* 141	% Retention (ret.) or incorporation (inc.) of ^{18}O in formate
[19-^{2}H]19-oxo (**4**,H* = D)	—	$^{16}O_2$	99·5	0·25	0·25	—
[19-^{2}H]19-oxo (**4**,H* = D)	—	$^{18}O_2$(99%)	5·4	90·0	4·6	90 (inc.)
[19-^{2}H]19-oxo (**4**,H* = D,O = ^{18}O)	61	$^{16}O_2$	47	49·8	2·2	82 (ret.)
[19*S*-^{2}H]19-hydroxy (**2**,H* = D	–	$^{16}O_2$	99·5	0·25	0·25	—
[19*S*-^{2}H]19-hydroxy (**2**,H* = D)	—	$^{18}O_2$(99%)	6·6	86·5	7·9	87 (inc.)
[19*S*-^{2}H;19-^{18}O]19-hydroxy (**2**,H* = D, O = ^{18}O)	62	$^{16}O_2$	41·9	56·1	2·0	90 (ret.)

† The deuterium label was introduced in order to obtain an accurate and realistic estimate of the ^{18}O content of the biosynthetic formate, because it was found necessary to minimise a contribution from endogenous unlabelled formate (*m/z* 136) and also from an isomeric impurity found at this mass (methyl benzoate).

protocol, that is the transformation of the ^{16}O-containing 19-hydroxy-compound (**2**) into oestrone (**5**) under $^{18}O_2$, the formate was found to contain 0·9 atom of ^{18}O, which in our view must have been incorporated during the terminal step (step 3, Scheme 1). Together, these experiments show that if a *gem*-diol of the type (**3**) is involved as an intermediate, then the hydroxy-group introduced in step 2a is the one eliminated in step 2b. Alternatively, the oxidation of the 19-hydroxy-group to the aldehyde could occur by a hitherto unrecognised facet of an activated oxygen species generated from NADPH and O_2.

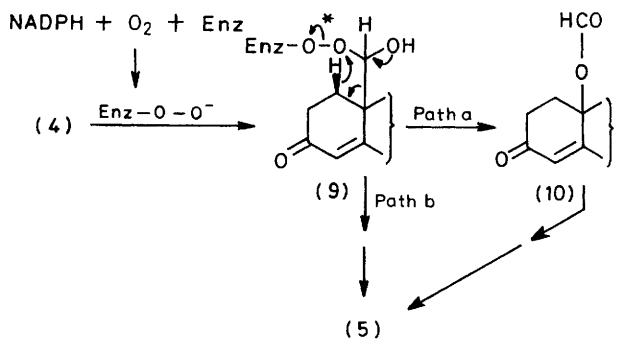
(iii) The initial hydroxylation reaction (step 1) converting androstenedione (**1**) into the 19-hydroxy-compound (**2**) has not yet been studied as a discrete entity, but the overall conversion of androstenedione (**1**; non deuteriated) into oestrone (**5**) was studied under $^{18}O_2$. Mass spectral analysis of the formate produced showed peaks at m/z 136 (impurity†) and 140, which corresponds to $HC^{18}O^{18}OH$. Absence of the species corresponding to $HC^{18}OOH$ proved that the demethylation process is attended by the incorporation of two oxygen atoms from O_2 into formate. These results from the overall reaction are thus fully in accord with the requirements of the stepwise study on the conversion of compounds (**2**) and (**4**) into (**5**). This is because the stepwise study predicts the formate to contain one oxygen atom introduced from O_2 during the initial hydroxylation reaction (step 1), and another in the final C(10)–C(19) bond cleavage reaction (step 3). The status of individual oxygen atoms in the overall transformation is thus shown in Scheme 1 by using open, half filled, and filled oxygen atoms for the individual steps.

The ^{18}O study reported above eliminates several mechanisms which have been proposed[6] for the cleavage of the C(10)–C(19) bond in oestrogen biosynthesis, and focuses attention on two broad mechanistic alternatives. The first alternative assumes that in step 3, NADPH and molecular O_2 are involved in a hydroxylation reaction producing either a 1β- or a 2β-hydroxy-intermediate. The hydroxy-group may then form a cyclic hemi-acetal of the type (**6**) or (**7**) (Scheme 2) with the C-19 carbonyl group. The hemi-acetal (**6**) would produce oestrone (**5**) by an electrocyclic rearrangement (mechanism 1a, Scheme 2), whereas (**7**) would proceed through a 2β-formyloxy-derivative (**8**) (mechanism 1b, Scheme 2). Although neither of these two mechanistic courses (1a and 1b, Scheme 2) are directly excluded by our experiments, the cyclisation process in mechanism 1a appears unlikely on stereochemical grounds. The second alternative (mechanism 2, Scheme 3)



SCHEME 2.

Mechanism 2



SCHEME 3. Arrows portray the course of path b.

rationalises the $^{18}O_2$ incorporation in the conversion of (**4**) into (**5**) by assuming that NADPH and O_2 participate in the formation of an enzyme bound 'peroxide' species which reacts with the aldehyde to give an intermediary complex [represented by the hypothetical structure (**9**)]. The latter species may then either produce oestrone (**5**) *via* a Baeyer-Villiger process in two steps (path a), or rearrange directly through a cyclic mechanism (path b). Path a, however, has been eliminated by the demonstration that 10β-hydroxyoestr-4-ene-3,17-dione 10-formate (**10**) is not converted into oestrone (**5**) by human placental microsomes.

In conclusion, the experimental data exclude all but mechanisms 1a, 1b, and 2b for the cleavage of the C(10)–C(19) bond in oestrogen biosynthesis. Of these, mechanism 1a may be regarded as unlikely on theoretical grounds, leaving two mechanisms (1b and 2b) to be evaluated by further experiments, though at present our preference is for mechanism 2b.

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