Mechanistic studies on C-19 demethylation in oestrogen biosynthesis

Muhammad AKHTAR, Michael R. CALDER, David L. CORINA and J. Neville WRIGHT
Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

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Mechanistic aspects of the biosynthesis of oestrogen have been studied with a microsomal preparation from full-term human placenta. The overall transformation, termed the aromatization process, involves three steps using O₂ and NADPH, in which the C-19 methyl group of an androgen is oxidised to formic acid with concomitant production of the aromatic ring of oestrogen:

To study the mechanism of this process in terms of the involvement of the oxygen atoms, a number of labelled precursors were synthesized. Notable amongst these were 19-hydroxy-4-androstene-3,17-dione (II) and 19-oxo-4-androstene-3,17-dione (IV) in which the C-19 was labelled with ²H in addition to ¹⁸O. In order to follow the fate of the labelled atoms at C-19 of (II) and (IV) during the aromatization, the formic acid released from C-19 was benzylated and analysed by mass spectrometry. Experimental procedures were devised to minimize the exchange of oxygen atoms in substrates and product with oxygens of the medium. In the conversion of the 19-[¹⁸O] compounds of types (II) and (IV) into 3-hydroxy-1,3,5-(10)-oestratriene-17-one (V, oestrone), it was found that the formic acid from C-19 retained the original substrate oxygen. When the equivalent ¹⁶O substrates were aromatized under ¹⁸O₂, the formic acid from both substrates contained one atom of ¹⁸O. It is argued that in the conversion of the 19-hydroxy compound (II) into the 19-oxo compound (IV), the C-19 oxygen of the former remains intact and that one atom of oxygen from O₂ is incorporated into formic acid during the conversion of the 19-oxo compound (IV) into oestrogen. This conclusion was further substantiated by demonstrating that in the aromatization of 4-androstene-3,17-dione (I), both the oxygen atoms in the formic acid originated from molecular oxygen. 10β-Hydroxy-4-oestrene-3,17-dione formate, a possible intermediate in the aromatization, was synthesized and shown not to be converted into oestrogen. In the light of the cumulative evidence available to date, stereochemical aspects of the conversion of the 19-hydroxy compound (II) into the 19-oxo compound (IV), and mechanistic features of the C-10–C-19 bond cleavage step during the conversion of the 19-oxo compound (IV) into oestrogen are discussed.

The multistep conversion of 4-androstene-3,17-dione (I) into 3-hydroxy-1,3,5-(10)-oestratriene-17-one (V) catalysed by the aromatase complex of human placenta (Ryan, 1959) has been the subject of extensive studies in this laboratory (Akhtar & Skinner, 1968; Skinner & Akhtar, 1969; Akhtar *et al.*, 1981). The results from our experiments, when taken in conjunction with those of Meyer (1955), Wilcox & Engle (1965) and Braselton *et al.*, (1973),

have been interpreted to suggest that the overall transformation proceeds through the involvement of three oxidation reactions, all requiring NADPH and O₂ as shown in Scheme 1 (Akhtar *et al.*, 1981, and references cited therein). The stoichiometry of this process was subsequently determined by Thompson & Siiteri (1974) who showed that the aromatization reaction, (I) to (V), indeed utilized 3 mol each of NADPH and O₂, thus providing further support for the pathway of Scheme 1. According to Scheme 1, the first hydroxylation occurs at C-19 to produce 19-hydroxy-4-androstene-3,17-dione (II) (Meyer,

Abbreviations used: TMS, trimethylsilyl; g.l.c.–m.s., gas liquid chromatography–mass spectrometry.

Scheme 1. Pathway showing the fate of individual oxygen atoms at C-19 during the conversion of 4-androstene-3,17-dione (I) into oestrogen (V)

1955); this, in the second oxidation step, is converted to the corresponding 19-aldehyde (IV), presumably through the intermediacy of the *gem*-diol (III) (Akhtar & Skinner, 1968). The mechanistic details and absolute stereochemistry of the second oxidation reaction, (II) to (IV), have been elucidated by a number of independent approaches (Arigoni *et al.*, 1975; Osawa *et al.*, 1975; Caspi *et al.*, 1978).

The third and final oxidation of (IV) is believed to provide the trigger for the cleavage of the C-10–C-19 bond to produce formic acid (Skinner & Akhtar, 1969) and the process is somehow linked to a stereospecific elimination of the 1β and 2β hydrogen atoms (Townsend & Brodie, 1968; Fishman *et al.*, 1969). We initiated a programme of research using ^{18}O -labelling (Akhtar *et al.*, 1976) to differentiate between the various mechanistic alternatives which have been considered in the literature for the bond-cleavage reaction of step 3, Scheme 1 (Hosoda & Fishman, 1974; Osawa & Shibata, 1974; Morand *et al.*, 1975; Akhtar *et al.*, 1976). Extending this approach, the present paper describes the sources of individual oxygen atoms incor-

porated into the C-19 demethylation product, formic acid, and discusses mechanisms which could account for our observations. A preliminary account of certain aspects of this study has been published (Akhtar *et al.*, 1981).

Experimental

Materials

Chemicals were purchased from BDH except for the following: oxygen gas $^{18}\text{O}_2$ (99.45 atom % excess) and NaB^2H_4 (99 atom % excess) (Prochem-B.O.C., London, U.K.); NADP^+ (Cambrian Chemicals, Croydon, U.K.); NaB^3H_4 (The Radiochemical Centre, Amersham, Bucks., U.K.); 3 β -hydroxy-5-androstene-17-one acetate and 5(10)-oestrone-3,17-dione (G. D. Searle and Co., Morpeth, U.K.).

Solvents were purified by redistillation; methanol was purified further by distillation from calcium oxide prior to use. Diazotoluene was prepared (Corina & Dunstan, 1973) from recrystallized *N*-benzyl-*N*-nitrosotoluene 4-sulphonamide (Over-

berger & Anselme, 1963) and after removal of solvent the diazotoluene was dissolved in dry tetrahydrofuran (1:4, v/v).

Synthesis of 19-[^{18}O]hydroxy-4-androstene-3,17-dione and 19-[^{18}O]oxo-4-androstene-3,17-dione (IIa and IVa, Scheme 2)

5-Bromo-3 β ,6 β -di[6- ^{18}O]hydroxy-5 α -androstan-17-one 3-acetate (VII, Scheme 2). *N*-Bromoacetamide (2g) was added in three portions at 10min intervals to a solution of 3 β -hydroxy-5-androstene-17-one acetate (VI) (2g) dissolved in dry dioxan (10ml) containing perchloric acid (sp.gr. 1.54; 10 μ l) and H_2^{18}O (70 atom % excess, 0.2ml). The reaction mixture was stirred in the dark at room temperature for a further 40min, and added to ice cold aq. Na_2SO_3 (6%, w/v; 150ml). The precipitate was filtered, dissolved in chloroform (25ml) and the organic layer was successively washed with saturated NaHCO_3 (3 $\times$ 25ml) and water (3 $\times$ 25ml). After drying over anhydrous Na_2SO_4 , the solvent was removed under reduced pressure, and the residual oil was crystallized from dichloromethane/light petroleum (b.p. 40–60°C) to give 5-bromo-3 β ,6 β -di[6- ^{18}O]hydroxy-5 α -androstan-17-one 3-acetate (VII); yield 1.3g. With respect to m.p., ^1H n.m.r., i.r. and t.l.c. mobility the above compound (VII) was shown to be identical with authentic unlabelled material (Akhtar & Barton, 1964).

5-Bromo-3 β -hydroxy-6 β ,19-[19- ^{18}O]epoxy-5 α -androstan-17-one 3-acetate (VIII). 5-Bromo-3 β ,6 β -di[6- ^{18}O]hydroxy-5 α -androstan-17-one 3-acetate (VII) (2g) was dissolved in dry benzene (350ml) and mixed with lead tetra-acetate (3.5g) and iodine (3.5g). The mixture was rapidly stirred and illuminated with a 275 W lamp (5cm away). After 6h the mixture was washed successively with aq. Na_2SO_3 (5%, w/v; 3 $\times$ 500ml) and water (3 $\times$ 250ml). After drying, the solvent was removed under reduced pressure and the product was crystallized from diethyl ether/light petroleum (b.p. 40–60°C) to give 5-bromo-3 β -hydroxy-6 β ,19-[19- ^{18}O]epoxy-5 α -androstan-17-one 3-acetate (VIII); yield 1.2g; m.p. and mixed m.p. with authentic 5-bromo-3 β -hydroxy-6 β ,19-epoxy-5 α -androstan-17-one 3-acetate, 187–188°C; 6 β ,19-oxide bridge characterized by a sharp medium-intensity band in i.r. between 903 and 910 cm^{-1} .

5-Bromo-6 β ,19-[19- ^{18}O]epoxy-5 α -androstan-3,17-dione (IX) was prepared from 5-bromo-3 β -hydroxy-6 β ,19-[19- ^{18}O]epoxy-5 α -androstan-17-one 3-acetate (VIII) by the method used for the unlabelled compound (Skinner & Akhtar, 1969).

19-[^{18}O]Hydroxy-4-androstene-3,17-dione (IIa). 5-Bromo-6 β ,19-[19- ^{18}O]epoxy-5 α -androstan-3,17-dione (IX) (0.8g) was refluxed in rapidly stirred isopropanol (50ml) containing zinc dust (6g) for 48h. Zinc was removed by centrifugation and washed with hot isopropanol (3 $\times$ 50ml). The com-

Scheme 2. Sequence of reactions for the synthesis of 19-hydroxy-4-androstene-3,17-dione and 19-oxo-4-androstene-3,17-dione and their C-19-labelled derivatives

bined isopropanol fractions were pooled, the volume was decreased to 50ml under reduced pressure, water (50ml) was added and the solution was extracted with chloroform (3 × 50ml). The organic extract was washed successively with 3 × 25ml each of dilute HCl, saturated NaHCO₃ and water. After drying over anhydrous Na₂SO₄, the solvent was removed under reduced pressure and the residue was crystallized from chloroform/diethyl ether to give 19-[¹⁸O]hydroxy-4-androstene-3,17-dione (IIa); yield 400mg; m.p. 176–178°C; u.v. (methanol) $\lambda_{\max}$ 243nm, ϵ 16218; mass spectrometric analysis of the TMS ether of 19-[¹⁸O]-hydroxy-4-androstene-3,17-dione (IIa) showed an ¹⁸O isotopic abundance of 64 atom % excess (from the ions at *m/z* 377 and 375). The ratio of ions *m/z* 103 and 105 ([CH₂OTMS]⁺; C-10–C-19 cleavage fragment; Braselton *et al.*, 1973) indicated an ¹⁸O abundance of 62 atom % excess, thus showing that compound (IIa) was regiospecifically labelled at C-19 to the extent of 97%.

19-[¹⁸O]Oxo-4-androstene-3,17-dione (IVa). 19-[¹⁸O]Hydroxy-4-androstene-3,17-dione (IIa) (100mg) was dissolved in dry dichloromethane (5ml) and pyridinium chlorochromate (130mg) suspended in dichloromethane (1.5ml) was added. The mixture was stirred at room temperature for 90min. The reaction mixture was poured into anhydrous diethyl ether (50ml) and filtered through a thin layer of magnesium trisilicate. The filtrate was successively washed with saturated NaHCO₃ (3 × 25ml) and water (3 × 25ml). After drying over anhydrous Na₂SO₄, ether was removed under reduced pressure. Crystallization of the residue from diethyl ether/light petroleum (b.p. 40–60°C) gave 19-[¹⁸O]oxo-4-androstene-3,17-dione (IVa); yield 60mg; the ¹⁸O content was 61 atom % excess from ratios of the ions at *m/z* 302 and 300.

Synthesis of 10 β -hydroxy-4-oestrone-3,17-dione formate (X)

10 β -Hydroxy-4-oestrone-3,17-dione. This was synthesized by the methods of Cross *et al.* (1964), and Colton (1956) except that *m*-chloroperbenzoic acid was used to convert 5(10)-oestrone-3,17-dione into 5,10 β -epoxy-5 β -oestrane-3,17-dione.

10 β -Hydroxy-4-oestrone-3,17-dione formate (X). Carbonyldiimidazole (0.57g) and formic acid (98–100%, w/v; 0.13ml) were mixed in dry chloroform (5ml). After 5 min, 10 β -hydroxy-4-oestrone-3,17-dione (100mg) was added and the mixture was left at room temperature for 72h. The solution was diluted further with chloroform (40ml), and successively washed with 3 × 25ml each of 0.1M-HCl, sat. NaHCO₃ and water. After drying over anhydrous Na₂SO₄, the solvent was removed under reduced pressure and the residual oil was applied to preparative silica-gel PF₂₅₄ plates, which were

developed in chloroform/ethyl acetate (4:1, v/v). Four compounds were separated: unchanged 10 β -hydroxy-4-oestrone-3,17-dione (*R_F* 0.19), 10 β -hydroxy-4-oestrone-3,17-dione formate (X) (*R_F* 0.35) and two less polar compounds (*R_F* 0.52 and *R_F* 0.71). 10 β -Hydroxy-4-oestrone-3,17-dione formate was eluted from silica gel with ethyl acetate/methanol (9:1, v/v). The solvent was removed under reduced pressure and the oil was crystallized by addition of diethyl ether to yield 23mg of compound (X); m.p. 149–152°C; $\nu_{\max}$ (Nujol) 1195, 1685 and 1745 cm⁻¹; ¹H n.m.r.: δ (p.p.m.) (100MHz, [²H]chloroform) 0.98 (3H,s, 18-CH₃), 5.96 (1H,s, 4-*H*) and 8.14 (1H,s, formate-*H*); u.v. (methanol), $\lambda_{\max}$ 239nm, ϵ 15900; [α]_D + 111 ± 1° (*c* 1.3 in chloroform); *M*⁺ = 316; (found: C, 71.90; H, 7.62; C₁₉H₂₄O₄ requires C, 72.13; H, 7.65).

The corresponding 10 β -hydroxy[G-³H]-4-oestrone-3,17-dione formate was prepared by the addition of ³H₂O (0.05ml, 250mCi) to the reaction mixture during the conversion of 5 β ,10 β -epoxy-oestrane-3,17-dione to 10 β -hydroxy-4-oestrone-3,17-dione. Subsequent esterification gave 10 β -hydroxy[G-³H]-4-oestrone-3,17-dione formate (sp. radioactivity 2.4 × 10⁶d.p.m. of ³H/mg).

Synthesis of (19S)-(19-²H₁) and (19S)-[19-³H₁] 19-hydroxy compounds

(19S)-19-Hydroxy(19-²H)-4-androstene-3,17-dione (IIc), (19S)-19-[¹⁸O]hydroxy(19-²H₁)-4-androstene-3,17-dione (IIb) and (19S)-19-hydroxy-[19-³H₁]-4-androstene-3,17-dione (IIa) were obtained by reduction of 19-[¹⁸O]oxo-4-androstene-3,17-dione (IVa) or 19-oxo-4-androstene-3,17-dione (IV) with the appropriate isotopically labelled sodium borohydride, by the following procedure.

To a solution of the appropriate 19-oxo compound (25mg) in methanol (2ml), NaB²H₄ (or NaB³H₄) (2mg) was added and after 1min water (2ml) was added. The mixture was extracted with dichloromethane (3 × 5ml) and the combined extracts were dried over anhydrous Na₂SO₄. The solvent was removed under reduced pressure and the product was crystallized from chloroform/light petroleum (b.p. 40–60°C) to give approx. 20mg of the appropriate C-19-labelled compound. Mass spectrometric analysis of the TMS ethers of the deuterated alcohols (IIc) and (IIb) showed isotopic abundances greater than 99 atom % excess ²H, measured at masses 374–375 (IIc) or 376–377 (IIb). Measurement of the ion intensities at *m/z* 103–104 from (IIc) and 105–106 from (IIb) [CH₂OTMS]⁺ (corresponding to the C-10–C-19 cleavage fragment, Braselton *et al.*, 1973) showed that all the ²H was located at the C-19 position. N.m.r. analysis (100MHz, [²H]chloroform) showed that 85% of the ²H label was in the 19S position as calculated from the ratio of areas of peaks at δ 4.05 and δ 3.90

(Arigoni *et al.*, 1975; Osawa *et al.*, 1975). Other physical constants were identical with those of authentic samples of 19-hydroxy-4-androstene-3,17-dione (II).

Synthesis of (19-²H) and [19-³H] 19-oxo compounds

19-Oxo(19-²H)-4-androstene-3,17-dione (IVc), 19-[¹⁸O]oxo (19-²H)-4-androstene-3,17-dione (IVb) and 19-oxo[19-³H]-4-androstene-3,17-dione (IVd) were prepared by pyridinium chlorochromate oxidation of the corresponding isotopically labelled 19-alcohols, using the method described in this Section for the oxidation of compound (II) to compound (IV).

Mass spectrometric analysis of the deuterated 19-oxo compounds (IVc) and (IVb) showed isotopic abundances of 70 and 63 atom % excess deuterium respectively, measured at masses 300–301 (IVc) and 302–303 (IVb). With respect to m.p., ¹H n.m.r. (except for decrease of the C-19 proton signal), i.r. and t.l.c. mobility, the compounds were identical with authentic 19-oxo-4-androstene-3,17-dione (IV).

Synthesis of ²HC¹⁸O₂H

Sodium formate (20 mg) was dissolved in 0.5 ml of 4 M-NaO²H in ²H₂O (99 atom % excess) and heated at 70°C for 17 h. After acidification to pH 4 with ²HCl in ²H₂O, formic acid was collected by lyophilization, neutralized (to Methyl Red) with 1 M-NaO²H in ²H₂O and lyophilized to give solid ²HCO₂Na, 98 atom % excess ²H. A saturated solution of ²HCO₂Na in 40 μl of H₂¹⁸O (99 atom % excess) was acidified to pH 3 with conc. HCl, sealed, and left at 30°C for 4 h. The solution was neutralized (to Methyl Red) and lyophilized to give approx. 6 mg of ²HC¹⁸O₂Na, 84 atom % excess ¹⁸O.

Measurement of radioactivity and t.l.c.

These were carried out as described previously (Akhtar *et al.*, 1978).

Preparation of placental microsomal fractions

The microsomal fraction was prepared as described previously (Akhtar *et al.*, 1980).

Incubation of 10β-hydroxy-4-oestrone-3,17-dione formate (X)

Full term placental microsomes (equivalent to 100 mg of protein) were suspended in 25 mM-Tris/maleic acid buffer, pH 7.4, (5.0 ml) supplemented with NADP⁺ (3 mM), glucose 6-phosphate (15 mM) and glucose 6-phosphate dehydrogenase (1 μkat/ml).

Following a 10 min preincubation period at 37°C, the microsomal suspension was divided into two 2.5 ml samples. To one sample, 19-oxo[G-³H]-4-androstene-3,17-dione (50 μg; 100 000 d.p.m. in 20 μl of methanol) was added; this incubation served

as a reference incubation to demonstrate microsomal aromatase activity. To the other sample, 10β-hydroxy[G-³H]-4-oestrone-3,17-dione formate (X) (50 μg; 120 000 d.p.m. in 20 μl of methanol) was added. After 10 min, each incubation mixture was extracted with ethyl acetate (3 × 5 ml), the pooled extracts from each incubation were dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The residue from each extraction was applied to silica-gel PF₂₅₄ plates, which were then developed in chloroform/methanol (25:1, v/v). Bands corresponding to both starting materials, and authentic samples of 3-hydroxy-1,3,5(10)-oestratriene-17-one (V) and 1,3,5(10)-oestratriene-3,17β-diol (Va) were removed and eluted with ethyl acetate. Analysis of the ³H content of each band showed that from the reference incubation of 19-oxo-[G-³H]-4-androstene-3,17-dione, 62% of the incubated radioactivity appeared in the combined oestrogen bands, whereas from the incubation of 10β-hydroxy[G-³H]-4-oestrone-3,17-dione formate (X) the combined oestrogen bands contained less than 5% of the incubated radioactivity. A band corresponding to unchanged 10β-hydroxy-4-oestrone-3,17-dione formate (X) retained 82% of the initial radioactivity.

Microsomal incubations under controlled atmospheres

Homogenized microsomes (equivalent to 250 mg of protein) were thawed, washed twice by repeated suspension in 25 mM-Tris/maleic acid buffer, pH 7.4, (40 ml) and centrifuged at 105 000 g for 45 min. The final microsomal pellet was suspended in 5 ml of the same buffer.

Incubation components were added to give the following final concentrations: NADP⁺ (3 mM), glucose 6-phosphate (15 mM), glucose 6-phosphate dehydrogenase (1 μkat/ml) and dithioerythritol (1 mM). The assay medium was immediately subjected to three alternating cycles of evacuation under vacuum and flushing with N₂ gas, then allowed to stand at room temperature for 10 min (to ensure reduction of NADP⁺). Incubations were initiated by the addition of the appropriate steroid substrate (500 μg) plus 19-³H-labelled tracer (100 000 d.p.m.) dissolved in methanol (25 μl). The whole system was finally subjected to a further three cycles of evacuation under vacuum and flushing with N₂ gas, before being sealed under the required ¹⁶O₂ or ¹⁸O₂ atmosphere. The identities of the substrates and the atmospheres under which they were incubated are shown in Tables 1 and 2.

Incubations were for 20 min at 37°C in a shaking water bath. Reactions were terminated by pouring the reaction medium into a 50 ml round-bottomed flask containing 1 ml of a solution of orthophosphoric acid (sp. gr. 1.75)/water (1:20, v/v).

Isolation, derivatization and analysis of formic acid

Immediately after acidification, the reaction mixture was frozen by immersion in liquid nitrogen and lyophilized. The acid-volatile distillate was collected in a liquid nitrogen trap. A portion of the total acid-volatile distillate was counted for radioactivity to determine the amount of [^3H]formic acid present. The remaining volatile fraction was adjusted to pH 8.0 by addition of 35 μl of 0.4 M-NaOH, converting the formic acid into the sodium salt. The volume of the sample was reduced to 0.25 ml by lyophilization, and it was transferred to a 1.0 ml glass vial and completely lyophilized. The content of each vial was dissolved in methanol (20 μl), the sodium formate converted back to formic acid by the addition of 12 μl of 1 M-HCl in tetrahydrofuran, and immediately esterified with 35 μl of diazotoluene solution to produce benzyl formate. Isotope analysis of the benzyl formate was performed on 3–5 μl aliquots, containing 2–8 μg of benzyl formate, by g.l.c.–m.s. (Corina, 1977).

Kinetic analysis of the aromatization of 19-hydroxy-4-androstene-3,17-dione (II)

19-Hydroxy[$\text{G-}^3\text{H}$]-4-androstene-3,17-dione (II) (600 μg , 1×10^6 d.p.m. in 25 μl of methanol) was added to 5.0 ml of the microsomal suspension containing NADP $^+$ (3 mM), glucose 6-phosphate (15 mM), glucose 6-phosphate dehydrogenase (1 $\mu\text{kat/ml}$) and dithioerythritol (1 mM). Aliquots of 0.5 ml were removed from the reaction flask at regular time intervals, and the steroid products were immediately extracted with ethyl acetate (3 $\times$ 5 ml). The extracts were dried over anhydrous Na_2SO_4 and solvent was removed under reduced pressure. The residue was redissolved in a minimum volume of ethyl acetate and chromatographed on preparative silica-gel PF $_{254}$ plates, developed in cyclohexane/ethyl acetate (1:1, v/v). The bands corresponding to authentic 19-hydroxy-4-androstene-3,17-dione (II), ($R_F = 0.21$); 19-oxo-4-androstene-3,17-dione (IV), ($R_F = 0.32$); 1,3,5(10)-oestratriene-3,17 β -diol (Va), ($R_F = 0.47$) and 3-hydroxy-1,3,5(10)-oestratriene-17-one (V), ($R_F = 0.6$), were removed and eluted with ethyl acetate. The eluates were dried and their radioactivity was determined.

Evaluation of isotopic exchange, and other control experiments

The microsomal incubation system described above, but with omission of the steroid substrates, and the subsequent isolation and analysis of formic acid, described above, were used in the control experiments. Exchange of ^{18}O from formic acid under the conditions of incubation and isolation was assessed by incubations (20 min) containing $^2\text{H}^{18}\text{O}^{18}\text{ONa}$ (50 μg). The ^{18}O content of the reisolated formic acid was determined by g.l.c.–m.s.

Levels of endogenous formic acid produced during the 20 min incubation period were determined by g.l.c.–m.s. measurements after extraction of incubations without added substrate. The possibility of an exchange between molecular oxygen and formic acid, mediated by the microsomes, was tested by incubating unlabelled formic acid with a microsomal preparation under $^{18}\text{O}_2$ gas for 20 min. No ^{18}O could be detected in the reisolated formic acid.

In order to evaluate the extent of ^{18}O exchange from the relatively labile C-19 aldehyde oxygen, 19-[^{18}O]oxo-4-androstene-3,17-dione (IVa, 500 μg in 25 μl of methanol) was incubated (20 min) in 5 ml of the microsomal system as described above. At 5 min intervals, 1 ml aliquots were removed and extracted with diethyl ether and the reisolated 19-[^{18}O]oxo-4-androstene-3,17-dione (IVa) was analysed for ^{18}O content by g.l.c.–m.s.

Results and discussion

Kinetic analysis of the aromatization of 19-hydroxy-4-androstene-3,17-dione (II)

A *prima facie* case for the involvement of a 19-functionalized steroid at the oxidation level of a 19-aldehyde (structure of type IV) in oestrogen biosynthesis existed prior to the investigation reported in this paper. The question whether, in the overall aromatization process (I) to (V), such a species participated as an enzyme-bound or free intermediate was, however a controversial issue (Braselton *et al.*, 1974; Osawa & Shibata, 1974). A resolution of this point was sought through the extension of the kinetic approach previously described by Wilcox & Engel (1965) and Braselton *et al.* (1974). The experiments in Fig. 1 show that in the conversion of 19-hydroxy[$\text{G-}^3\text{H}$]-4-androstene-3,17-dione (II) into 3-hydroxy-1,3,5(10)-oestratriene-17-one (V), 19-oxo-4-androstene-3,17-dione (IV) was detected at 30 s as the sole metabolite. At 2 min after the initiation of the incubation (Fig. 2) the level of 19-oxo-4-androstene-3,17-dione (IV) was shown to be 8-fold that of 3-hydroxy-1,3,5(10)-oestratriene-17-one (V). After reaching a maximum concentration at 5 min, the amount of 19-oxo-4-androstene-3,17-dione (IV) declined with the concomitant accumulation of oestrogens (V) and (Va). These results, together with earlier observations (Braselton *et al.*, 1974; Wilcox & Engel, 1965) prove that in the aromatization of 19-hydroxy-4-androstene-3,17-dione (II), and hence, by implication, of the physiological substrate 4-androstene-3,17-dione (I), a species at the oxidation level of an aldehyde participates as an intermediate. The ^{18}O experiments described in a later section suggest that the species released into the medium may be the aldehyde itself and not its hydrated form.

Fig. 1. Radiochromatogram scans of t.l.c. plates showing the initial appearance (0–2 min) of radioactivity in 19-oxo-4-androstene-3,17-dione (B) and 3-hydroxy-1,3,5(10)-oestratriene-17-one (C) on incubation of 19-hydroxy[$G-^3H$]-4-androstene-3,17-dione (A) with placental microsomes

Experiments with ^{18}O and 19-hydroxy and 19-oxo-4-androstene-3,17-dione

The experiments with ^{18}O reported in this paper fall into two broad categories. The first involved the aromatization of the two C-19 functionalized intermediates, (II) and (IV), under an atmosphere of $^{18}O_2$, and the second in which their ^{18}O -labelled counterparts (IIa) and (IVa) were subjected to the same biological transformation but under $^{16}O_2$. In both types of experiments, the status and origin of oxygen atoms during the aromatization process was evaluated through the analysis of formate released from C-19. Isolation and derivatization of the biosynthetic formate under conditions maintaining the integrity of its two relatively labile oxygen atoms posed several technical problems; the resolution of these is described in detail in the Experimental section.

Fig. 2. Quantitative measurement of 19-oxo-4-androstene-3,17-dione and oestrogens (V and Va) after incubation of 19-hydroxy[$G-^3H$]-4-androstene-3,17-dione with placental microsomes

Compounds were eluted and quantified by scintillation counting after separation by t.l.c. ●, 19-oxo(IV); ○, combined oestrogens (V and Va).

The results in Table 1 show that in the conversion of 19-hydroxy-4-androstene-3,17-dione (II) and 19-oxo-4-androstene-3,17-dione (IV) into 3-hydroxy-1,3,5(10)-oestratriene-17-one (V) under an atmosphere of $^{18}O_2$, 0.50 and 0.70 atom respectively from molecular oxygen were incorporated into formate per molecule of product. The transfer of ^{18}O from the two labelled substrates (IIa) and (IVa) into formate was also convincingly demonstrated, but the amount transferred was again a fractional number, being 0.54 and 0.66 atom respectively. These results, though encouraging, were subject to ambiguous interpretation and therefore necessitated further investigation. Two types of interfering processes which could lead to an underestimation of the ^{18}O -content of the formate were recognized.

The first was the loss of ^{18}O from the steroid substrate as well as from formate due to exchange with oxygen atoms of the medium. The experimental parameters were manipulated to find conditions under which the extent of ^{18}O exchange from substrates and product was minimal. The least exchange from 19-[^{18}O]oxo-4-androstene-3,17-dione was found for incubations conducted in Tris/maleic acid buffer, in which the loss of ^{18}O did not exceed 25% over a 20 min incubation period. Under the chosen incubation conditions as described in the Experimental section, no loss of ^{18}O was observed from recovered 19-[^{18}O]hydroxy-4-androstene-3,17-dione, while small losses (<4%) from [^{18}O]formic acid, reisolated from microsomal incubations, occur-

Table 1. ^{18}O content of formate released from C-19 during oestrone biosynthesis using non-deuterated substrates 4-Androstene-3,17-dione, unlabelled or ^{18}O -labelled 19-oxo-4-androstene-3,17-dione and 19-hydroxy-4-androstene-3,17-dione were incubated with placental microsomes under atmospheres of $^{16}\text{O}_2$ or $^{18}\text{O}_2$ as described in the Experimental section. The C-19 demethylation product, formic acid, was isolated and converted into benzyl formate, in which the ^{18}O content was measured by g.l.c.-m.s. Roman numerals refer to the structures in Scheme 1.

Substrate	^{18}O atom % excess in substrate	Gas phase	Relative intensities of peaks due to benzyl ester of:			Retention (ret) or incorporation (inc) of ^{18}O in formate (%)
			HCO_2H (m/z 136)	HC^{18}OOH (m/z 138)	$\text{HC}^{18}\text{O}^{18}\text{OH}$ (m/z 140)	
19-Oxo (IV)	—	$^{16}\text{O}_2$	99.5	0.25	0.25	—
19-Oxo (IV)	—	$^{18}\text{O}_2$ (99%)	29.0	70.0	1.0	70 (inc)
19-[^{18}O]Oxo (IVa)	60	$^{16}\text{O}_2$	60.4	39.6	1.0	66 (ret)
19-Hydroxy (II)	—	$^{16}\text{O}_2$	99.5	0.25	0.25	—
19-Hydroxy (II)	—	$^{18}\text{O}_2$ (99%)	50.1	49.9	1.0	50 (inc)
19-[^{18}O]Hydroxy (IIa)	60	$^{16}\text{O}_2$	67.4	32.6	1.0	54 (ret)
4-Androstene-3,17-dione (I)	—	$^{18}\text{O}_2$ (99%)	44.7	9.08	46.2	Ratio of $\text{HC}^{18}\text{O}^{18}\text{OH}/\text{HC}^{18}\text{OOH} = 5:1$

Table 2. ^{18}O content of the formate released from C-19 during oestrone biosynthesis using deuterated substrates Variously-labelled 19-oxo-4-androstene-3,17-dione and 19-hydroxy-4-androstene-3,17-dione were incubated with placental microsomes under atmospheres of $^{16}\text{O}_2$ or $^{18}\text{O}_2$ as described in the Experimental section. The C-19 demethylation product, (^2H) formic acid, was isolated and converted into benzyl formate in which the ^{18}O content was measured by g.l.c.-m.s. Roman numerals refer to the structures in Scheme 1.

Substrate		¹⁸ O atom % excess in substrate	Gas phase	Relative intensities of peaks due to benzyl ester of:			Retention (ret) or incorporation (inc) of ¹⁸ O in formate (%)
				² HCO ₂ H (<i>m/z</i> 137)	² HC ¹⁸ OOH (<i>m/z</i> 139)	² HC ¹⁸ O ¹⁸ OH (<i>m/z</i> 141)	
Single ¹⁸ O-labelling							
<i>a</i>	19-Oxo(19- ² H) (IVc)	—	¹⁶ O ₂	99.5	0.25	0.25	—
<i>b</i>	19-Oxo(19- ² H) (IVc)	—	¹⁸ O ₂ (99%)	5.4	90.0	4.6	90 (inc)
<i>c</i>	19-[¹⁸ O]Oxo(19- ² H) (IVb)	61	¹⁶ O ₂	47.0	49.8	2.2	82 (ret)
<i>d</i>	(19 <i>S</i>)-19-Hydroxy(19- ² H ₁) (IIc)	—	¹⁶ O ₂	99.5	0.25	0.25	—
<i>e</i>	(19 <i>S</i>)-19-Hydroxy(19- ² H ₁) (IIc)	—	¹⁸ O ₂ (99%)	6.6	86.5	7.9	87 (inc)
<i>f</i>	(19 <i>S</i>)-19-[¹⁸ O]Hydroxy(19- ² H ₁) (IIb)	62	¹⁶ O ₂	41.9	56.1	2.0	90 (ret)
Twin ¹⁸ O-labelling							
<i>g</i>	19-[¹⁸ O]Oxo(19- ² H) (IVb)	61	¹⁸ O ₂ (99%)	5.8 (4.7)	53.1 (47.3)	41.1 (45.0)	Found Predicted*
<i>h</i>	(19 <i>S</i>)-19-[¹⁸ O]Hydroxy(19- ² H ₁) (IIb)	62	¹⁸ O ₂ (99%)	6.8 (5.4)	43.8 (43.8)	49.4 (48.8)	Found Predicted*

* Values in parentheses are those predicted from single ^{18}O labelling experiments of the type (b plus c, or e plus f).

red predominantly during extraction and esterification. In the aromatization of both 4-androstene-3,17-dione (I) and the 19-hydroxy compound (II), the 19-aldehyde (IV) is an obligatory intermediate. Though the lifetime of, and hence the isotopic exchange rate for, the C-19 carbonyl oxygen of (IV) are not known, some loss of the isotopic oxygen must occur at this stage when ^{18}O labelling studies are conducted using (I) and (II) as substrates. The experimental values reported for the ^{18}O content of formic acid in Tables 1 and 2 are thus an underestimation, because they have not been corrected for the various exchanges referred to above.

The second interfering process could be a dilution of the biosynthetic formate with the non-isotopic species ubiquitously present [the latter phenomenon would underestimate the ^{18}O -content since this value is calculated from the ratio of peak intensities due to fragments m/z 136 and 138, originating from the benzyl ester derivatives of HCOOH and HC^{18}OOH]. Blank incubations of microsomes, on extraction and mass spectral analysis, indeed gave a peak at m/z 136, attributed to the benzyl ester of endogenous formic acid, and also to an isomeric impurity of methyl benzoate. To circumvent this interference in the determination of the true ^{18}O

value, it was decided to employ deuterated substrates, in both the ^{16}O and ^{18}O series. The C_1 unit now would be eliminated as $^2\text{HCOOH}$ and $^2\text{HC}^{18}\text{OOH}$, giving rise to molecular ions at m/z 137 and 139 for their corresponding benzyl esters in a clear region of the spectrum.

Experiments with ^{18}O and 19-oxo(19- ^2H)-4-androstene-3,17-dione

In order to study the fate of molecular oxygen in the reaction of step 3, Scheme 1, the deuterated aldehyde derivative (IVc) was aromatized under an atmosphere of $^{18}\text{O}_2$ and the C-19 carbon atom was isolated as formic acid and analysed for ^{18}O . The results in Table 2 (Expt. *b*) show that 90% of the molecules of (^2H)formate released during the enzymic conversion contained 1 atom of oxygen derived from $^{18}\text{O}_2$ gas. The data show a considerably higher incorporation of ^{18}O compared with that obtained either using non-deuterated substrates (Table 1) or from experiments reported previously (Akhtar *et al.*, 1976) in which the protium analogue (IV) of the aldehyde, generated *in situ*, was used for the biosynthetic experiments. Next, the fate of the aldehydic oxygen was followed using 19-[^{18}O]-oxo(19- ^2H)-4-androstene-3,17-dione (IVb) as substrate; in the transformation of this substrate into 3-hydroxy-1,3,5(10)-oestratriene-17-one (V) under $^{16}\text{O}_2$, the aldehydic oxygen was retained in the formate to the extent of 82% (Table 2, Expt. *c*). Additional 'twin' labelling experiments in which the 19-[^{18}O]aldehyde (IVb) was incubated under an atmosphere of $^{18}\text{O}_2$ gave formic acid exhibiting an ^{18}O labelling pattern expected from the summation of the values found for the corresponding single-labelling experiments (Table 2, compare Expt. *g* with Expts. *b* and *c*). These results confirm that the formic acid produced during the conversion of (IV) to (V) contains one oxygen derived from the C-19 carbonyl oxygen atom of (IV) and the other from molecular oxygen. Attention is drawn to the fact that the retention of the ^{18}O from 19-[^{18}O]oxo(19- ^2H)-4-androstene-3,17-dione (IVb) into formic acid proves that the C-19 carbonyl group of compound (IV) neither participates in Schiff-base formation nor is transferred to a carrier, such as tetrahydrofolate, during the cleavage reaction.

Experiments with ^{18}O and (19S)-19-hydroxy(19- $^2\text{H}_1$)-4-androstene-3,17-dione

The oxidation of the primary hydroxy group in the compound (II) to an aldehyde group in compound (IV) is dependent on the involvement of NADPH and molecular oxygen (Akhtar & Skinner, 1968). To explain this observation it was proposed that a *gem*-diol, of the type shown in structure (III) of Scheme 1, may be involved in an intermediary role in this process. The *gem*-diol (III) would then

undergo a dehydration step to give the 19-aldehyde (IV).

To investigate this step, the aromatization of (19S)-19-hydroxy(19- $^2\text{H}_1$)-4-androstene-3,17-dione (IIc) under $^{18}\text{O}_2$ gas, and (19S)-19-[^{18}O]hydroxy(19- $^2\text{H}_1$)-4-androstene-3,17-dione (IIb) under $^{16}\text{O}_2$ gas, was studied. Analysis of the formate produced (Table 2, Expt. *f*) showed that the hydroxyl oxygen atom of (19S)-19-[^{18}O]hydroxy(19- $^2\text{H}_1$)-4-androstene-3,17-dione (IIb) was transferred to formate to the extent of 90%, thus implying that in the conversion of the alcohol (IIb) into aldehyde (IVb) the original oxygen of the former is retained. In the opposite protocol, that is the transformation of the ^{16}O -containing 19-hydroxy compound (IIc) into 3-hydroxy-1,3,5(10)-oestratriene-17-one (V) under $^{18}\text{O}_2$, each molecule of formate was found to contain 0.9 atom of ^{18}O (Table 2, Expt. *e*) which in our view must have been incorporated during the terminal step (step 3 of Scheme 1). A 'twin' labelling experiment also was performed in this series by incubating (IIb) under $^{18}\text{O}_2$ gas. A similar correspondence with predicted values was observed (Table 2, Expt. *h*) as found for the 19-oxo series.

Together, these experiments show that if a *gem*-diol of the type (III) is involved in an intermediary role in this process, then the hydroxyl group introduced in step 2*a* is the one eliminated in step 2*b* (Scheme 1). Alternatively, the oxidation of the 19-hydroxy group to an aldehyde could occur by a hitherto unrecognized facet of an activated oxygen species generated from NADPH and oxygen.

Experiments with ^{18}O and 4-androstene-3,17-dione (I)

The initial hydroxylation reaction (step 1) converting 4-androstene-3,17-dione (I) into 19-hydroxy-4-androstene-3,17-dione (II) has not yet been studied as a discrete entity, but the overall conversion of 4-androstene-3,17-dione (I, non-deuterated) into 3-hydroxy-1,3,5(10)-oestratriene-17-one (V) was studied under $^{18}\text{O}_2$. Mass spectral analysis of the biosynthetic formate showed a peak at m/z 136 attributed to endogenous formate and two other peaks due to [$^{18}\text{O}_1$]formate and [$^{18}\text{O}_2$]formate at m/z 138 and 140 respectively. The fact that the signal corresponding to $\text{HC}^{18}\text{O}^{18}\text{OH}$ was at least five times stronger than for $\text{HC}^{18}\text{O}^{16}\text{OH}$ proves that the overall demethylation process in oestrogen biosynthesis is attended by the incorporation of two oxygen atoms from molecular oxygen into formate.

The results from the study of the overall reaction are thus fully in accord with the requirements of the step-wise conversion of compounds (II) and (IV) into (V). This is because the step-wise process predicts that the formate would contain one oxygen atom introduced from O_2 during the initial hydroxylation reaction (step 1), and one oxygen atom intro-

duced 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.

Possible involvement of 10 β -hydroxy-4-oestrene-3,17-dione formate (X) in oestrogen biosynthesis

One of the possible mechanisms (mechanism 2, path *a*, Scheme 6) for the final oxidative step (step 3) in oestrogen biosynthesis entails the involvement of 10 β -hydroxy-4-oestrene-3,17-dione formate (X). To examine this possibility, compound (X) labelled with ^3H was synthesized and incubated with placental microsomes; no significant formation of oestrogens could be detected. Full details of this and also of an appropriate control experiment are described in the Experimental section.

Conclusion and mechanistic considerations

The biosynthesis of oestrogen in human placenta may be regarded as commencing from the C₁₉ steroid, 3 β -hydroxy-5-androstene-17-one (XV, R = H). This precursor, in the form of a sulphate conjugate (XVa, R = SO₃), is made available to the tissue through the co-operative action of maternal liver and foetal adrenal. The formation of the free steroid (XV, R = H; Scheme 3) from the conjugate is catalysed by a sulphatase present in abundance in the placenta. The elaboration of the oestrogen ring system from (XV, R = H) is achieved in two stages: anaerobic and aerobic. In the anaerobic stage the Δ^5 -3-hydroxy system of (XV, R = H) is converted into the Δ^4 -3-oxo functionality of (I) and the mechanistic details for this process have been reported in a previous publication (Akhtar *et al.*, 1980).

The demethylation of the Δ^4 -3-oxo steroid (I) to produce oestrogen (V) constitutes the aerobic stage and involves the participation of three oxygen-dependent reactions as shown in Scheme 1. Several

facets of these reactions have been studied in this paper.

The kinetic data in Fig. 1, when taken in conjunction with the results from experiments using ^{18}O (Tables 1 and 2) show that the 19-aldehyde (IV) is a free intermediate in the demethylation process. In the aromatization of the 19-hydroxy compound (II) the formation of the 19-aldehyde (IV) could be convincingly demonstrated before the aromatic product (V) was detectable. This may be interpreted to suggest that the conversion of the 19-hydroxy compound (II) into the 19-aldehyde (IV) (step 2, Scheme 1) and the C-10–C-19 bond cleavage reaction of step 3, are catalysed at two different active sites. If these two reactions occurred at the same active site then the 19-aldehyde (IV), or its equivalent *gem*-diol (III), would be expected to exist only in an enzyme-bound form and hence would be undetectable by the techniques used in this study. The argument that the dissociation constant for the aldehyde–enzyme complex may be high enough to permit the escape of this intermediate into the medium, though appearing attractive at first sight, is untenable since even under such a circumstance the formation of the aromatic product (V) alongside the aldehyde (IV) would be expected during the early stages of the reaction. This was not the case.

We now consider the exact chemical nature of the species that is released in the reaction of step 2, Scheme 1 and utilized as a substrate in the reaction of step 3, Scheme 1. The fact that ^{18}O from the 19-[^{18}O]hydroxy compound (IIb) was almost quantitatively recovered in the biosynthetic formate proves that if a *gem*-diol of the type (III) is an intermediate in this transformation, its dehydration must occur stereospecifically and hence enzymically. This necessitates that the 19-aldehyde (IV) be the product that is released in the reaction of step 2, Scheme 1. Similarly the high incorporation of ^{18}O from the 19-[^{18}O]aldehyde (IVb) into the formate suggests that the 19-aldehyde, and not its hydrated form produced non-enzymically, is the entity that is removed from the medium for utilization as substrate in the conversion of step 3, Scheme 1. This argument once again is based on the stereochemical consideration that non-enzymic hydration would produce racemic *gem*-diols requiring only 50% of the ^{18}O of the 19-[^{18}O]aldehyde (IVb) to be retained in the biosynthetic formate, against 80% experimentally found. This assertion must not detract from the fact that a non-enzymic hydration–dehydration phenomenon of the type below (Scheme 4) may occur as a side reaction under our conditions and account for the discrepancy which exists between the ^{18}O content of the steroid substrates and that in the biosynthetic formate.

In the literature several mechanisms have been considered for the cleavage of the C-10–C-19 bond

Scheme 3. Structure of 3 β -hydroxy-5-androstene-17-one and its sulphate conjugate

Scheme 4. Racemization of the ^{18}O label at C-19 through non-enzymic hydration-dehydration of the 19-aldehyde

in oestrogen biosynthesis. Some of these mechanisms can be, and indeed have been, modified to accommodate our original suggestions that one atom from O_2 is incorporated into formate during the reaction of step 3, Scheme 1. However, we regard only two of the broad proposals as deserving serious consideration (Schemes 5 and 6).

The first assumes that in step 3, NADPH and molecular O_2 are involved in a hydroxylation reaction producing either a 1β - or 2β -hydroxy intermediate (Scheme 5; XI, XII) (Townsend & Brodie, 1968; Fishman & Raju, 1981). The mechanisms (Scheme 5; 1a and 1b) rationalizing the formation of an aromatic ring from these intermediates (XI, XII) require that the hydroxyl groups are eliminated as water. (For the removal of 1β - and 2β -hydroxy groups, see Townsend & Brodie, 1968 and Hosoda & Fishman, 1974, respectively.) The

demonstration in the present paper that the O_2 participating in the final stage of the aromatization process contributes one atom of oxygen to formate necessitates revision of the previous mechanisms (Osawa & Shibata, 1974; Morand *et al.*, 1975; Fishman & Raju, 1981). Retaining the basic tenets of the original proposals, Scheme 5 presents a modified view in which the 1β - or 2β -hydroxy groups react with the C-19 carbonyl, producing the hemiacetals (XIa) and (XIIa) respectively. Formate is then produced from hemiacetal (XIa), in a single step by an electrocyclic rearrangement (Scheme 5; mechanism 1a). Chemical considerations necessitate that in the case of the hemiacetal (XIIa), this process would occur in two steps via the intermediacy of a 2β -formyloxy derivative of the type (XIII) (mechanism 1b).

Although neither of the mechanistic courses 1a and 1b represented in Scheme 5 are directly excluded by our experiments, the cyclization process (XI)→(XIa) appears unlikely on the grounds of the unfavourable energetics involved in the formation of a 4-membered ring. An unpalatable feature of the theory, which advocates the role of a 2β -hydroxy compound of the type (XII) in the aromatization process, is the assumption that the crucial transformation (XII)→(V) in the biosynthesis of a key hormone occurs non-enzymically (Fishman & Raju, 1981).

Scheme 5. Mechanistic alternatives for the C-10-C-19 bond cleavage step involving hydroxylated intermediates

Mechanism 2

Scheme 6. Mechanistic alternatives for the C-10-C-19 bond cleavage step using an enzyme-bound peroxide intermediate

The arrows in structure (XIV) denote path b.

The second broad mechanistic alternative (mechanism 2; Scheme 6) rationalizes the incorporation of oxygen from ¹⁸O in the conversion of (IV) into (V) by assuming that NADPH and O₂ participate in the formation of an enzyme bound 'peroxide' species which reacts with the carbonyl group of the aldehyde to give an intermediary complex [represented by the hypothetical structure of the type (XIV)]. Aromatization of the latter species (XIV) may proceed via a Baeyer-Villiger type of process either in two steps (path a), or directly through a cyclic mechanism (path b) in Scheme 6. The participation of a two-step mechanism represented by path a, however, has been rendered unlikely by the demonstration that the 10β-formate (X) is not aromatized by human placental microsomes. Thus in conclusion, the experimental data available to date exclude all but mechanisms 1a, 1b and 2b for the cleavage of the C-10-C-19 bond in the course of oestrogen biosynthesis. Of these, mechanism 1a may be regarded as unlikely on theoretical grounds, leaving two mechanisms to be evaluated by further experiments.

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