Incorporation of Label from $^{18}O_2$ into Acetate during Side-chain Cleavage Catalysed by Cytochrome P-450$_{17\alpha}$ (17α-hydroxylase-17,20-lyase)

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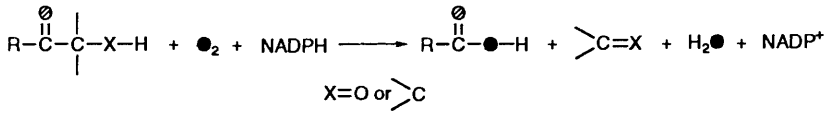
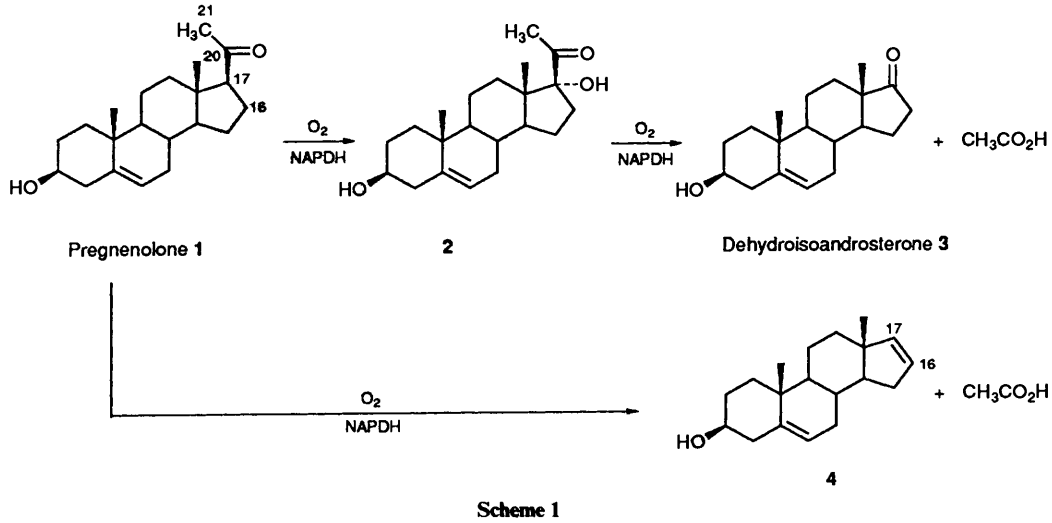
Samples of pregnenolone and 17α-hydroxypregnenolone deuteriated at the C-21 methyl group have been prepared and subjected to side-chain cleavage with a pig testes microsomal preparation under $^{18}O_2$. 17α-Hydroxypregnenolone was exclusively cleaved to dehydroisoandrosterone and the acetic acid released during the process was found to incorporate 0.90 atom of ^{18}O. When a similar incubation was performed with pregnenolone two steroidal products, dehydroisoandrosterone and androsta-5,16-dien-3β-ol, were formed in an approximate ratio of 1:2–3 and the acetic acid formed in the process was again shown to contain 0.85–0.90 atom of ^{18}O. Complementary experiments in which the two substrates labelled with ^{18}O at the C-20 carbonyl group were incubated under $^{16}O_2$ gave acetic acid retaining between 65–85% of the original carbonyl oxygen. The experiments strengthen the hypothesis that the two C(17)–C(20) bond cleavages described above correspond to the acyl–carbon fission process of the equation below showing the indicated fate of the various oxygen atoms:



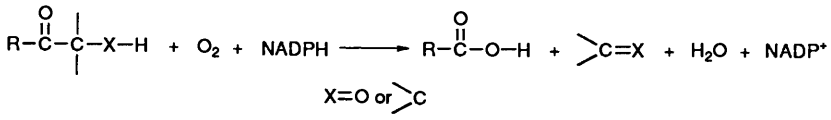
Recent mechanistic studies on sterol and steroid hormone biosynthesis together with advances in the purification of enzymes involved in the process have highlighted that certain cytochrome P-450 dependent oxygenases are involved not only in the conventional hydroxylation process [eqn. (1)], but also in other generic reactions, most noteworthy of which are the

$$R–H + O_2 + NADPH \longrightarrow ROH + H_2O + NADP \quad (1)$$

cleavage of C–C bonds.[1–4] An example of this is 17α-hydroxylase-17,20-lyase (also known as P-450$_{17\alpha}$) which catalyses the removal of the pregnenolone side-chain **1** (Scheme 1) to produce dehydroisoandrosterone **3**. The enzyme not only promotes[5] the initial hydroxylation to give 17α-hydroxypregnenolone **2** but also its further conversion into **3**. The enzyme also possesses another activity,[6] which culminates in the formation of the Δ^{16}-compound **4**. A third reaction resulting in the formation of a 17α-hydroxyandrogen has also been attributed to the lyase[7,8] and this aspect is the subject of a detailed study in a subsequent publication. We have suggested[1–4] that the C–C bond cleavage catalysed by the lyase as well as other related enzymes such as aromatase and 14α-demethylase, corresponds to an acyl–carbon cleavage and may be represented by the general reaction of Scheme 2. Although a diverse range of mechanisms are possible for the rationalisation of the reaction of Scheme 2, the two most likely options use either Fe^{III}–OOH or Fe^{IV}–O$^{\bullet}$↔(+$^{\bullet}$)Fe^{IV}=O species in a crucial role. In the light of our extensive studies on aromatase[1,3,9] these mechanisms postulate that the reaction of Scheme 2 is attended by the incorporation of an atom of oxygen from O_2 into the cleaved fragment. The present paper scrutinises this possibility through the study of two pregnene side-chain cleavages



Scheme 1



Scheme 2

Table 1 ^{18}O Content of acetate following cleavage of the C(17)–C(20) bond of isotopically labelled substrates by neonatal porcine testicular microsomes[a]

Substrate	Gas phase	Relative areas (%) of peaks due to the benzyl esters of: C^2H_3COOH (*m/z* 153)	$C^2H_3C^{18}OOH$ (*m/z* 155)	$C^2H_3C^{18}O^{18}OH$ (*m/z* 157)
[17α,21-$^2H_4$]Pregnenolone	$^{16}O_2$	99	1	—
[17α,21-$^2H_4$]Pregnenolone	$^{18}O_2$	5.05	91.20	3.75
[16α,17α,21-$^2H_5$]Pregnenolone	$^{18}O_2$	7.5	85.4	7.1
[21-$^2H_3$]-17α-Hydroxypregnenolone	$^{18}O_2$	9.05	90.40	0.55
[21-$^2H_3$;20-^{18}O]-17α-Hydroxypregnenolone	$^{16}O_2$	13	87	—
[16α,17α,21-$^2H_5$;20-^{18}O]Pregnenolone	$^{16}O_2$	34	66	—

[a] The results under entries 1–4 were reproduced in at least a dozen independent experiments; those in entries 5 and 6 in four different incubations. The precise isotopic composition of doubly labelled pregnenolone and 17α-hydroxypregnenolone samples is given in the Experimental section. In all cases, the isotopomers of acetate containing three deuterium atoms were the predominant species and, for clarity, the intensities of peaks only due to these are included in the calculations.

catalysed by 17α-hydroxylase-17,20-lyase from neonatal pig testes. A preliminary account of a part of this work has been published.[10]

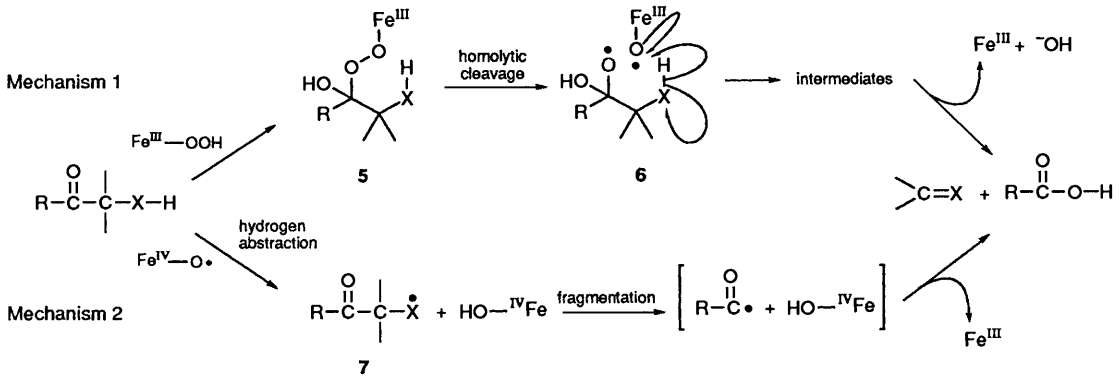
Results and Discussion

The key requirement of our approach was to determine accurately the isotopic content of the carboxy oxygen atoms of acetic acid[11] released during the side-chain cleavage process. Since acetic acid is ubiquitously present in buffers and in enzyme preparations, it was important to evolve a strategy in which the analytical method viewed only those acetic acid molecules which had specifically originated from the cleavage during the course of the *in vitro* incubations. For this purpose, the two substrates used in the present study, pregnenolone **1** and 17α-hydroxypregnenolone **2**, were labelled at position 21 with deuterium, so that the deuteriated isotopomers of acetic acid may be targeted for quantitation. Furthermore, in order to ensure that the carboxylic oxygen atoms of acetic acid did not undergo exchange with H_2O under various manipulations, the acetic acid was isolated from incubation mixtures by freeze-drying following gentle acidification. The distillate was then neutralised with NaOH and the solution again freeze-dried to obtain sodium acetate. The latter was then acidified and immediately treated with diazotoluene to give benzyl acetate. The resulting sample was subjected to GC–MS analysis and the various isotopomers of benzyl acetate quantified by monitoring the intensities of ions from *m/z* 150 to *m/z* 157; which correspond to the molecular ions of unlabelled benzyl acetate and its heavier isotopomers. As mentioned above, for quantitation the intensities of only those ions which contained three deuterium atoms (*m/z* 153 to 157) were considered.

[21-$^2H_3$]-17α-Hydroxypregnenolone (0.3 μmol cm^{-3}) was mixed with a tracer quantity of the corresponding species containing ^3H at C-21, and incubated under $^{18}O_2$ with a microsomal enzyme preparation (10 mg of protein per cm^3) obtained from neonatal pig testes[5] in the presence of an NADPH regenerating system for 30–120 min. The incubation mixture was processed to obtain the C-20 and C-21 of the precursor as acetate and from the measurement of ^3H used as the tracer, it was found that under the incubation conditions *ca.* 65% of the precursor had undergone side-chain cleavage. The analysis of the steroid fraction showed that dehydroisoandrosterone was the only product formed. The acetic acid obtained from the volatile fraction, following conversion into benzyl acetate,[9] was analysed by GC–MS to show that the predominant species contained three deuterium atoms and one atom of ^{18}O (*m/z* 155) from molecular oxygen. By comparing the intensities of molecular ion peaks due to $^2H_3CCOOBzl$ and $^2H_3CC^{18}OOBzl$ it was found that the incorporation of 0.91 atom of ^{18}O into the acetate occurred in the side-chain cleavage. These results are in accord with the general mechanism for P-450 dependent lyases proposed by us[3,4,10] and the incorporation of an atom of molecular oxygen into acetate during the formation of the 17-keto-steroid as originally predicted by Lynn and Brown.[11]

Next, a similar incubation under $^{18}O_2$ was performed using [16α,17α,21-$^2H_5$]pregnenolone. This labelling pattern was chosen to obtain quantitative information on the incorporation of ^{18}O into the side-chain released as acetate as well as the steroid nucleus. It was found that the major isotopomer of acetate contained three deuterium atoms and 92% of such molecules were also labelled with one atom of ^{18}O (*m/z* 155). We have already seen above that in the cleavage of the side-chain of 17α-hydroxypregnenolone when the exclusive steroid formed is dehydroisoandrosterone, the released acetate contained 0.91 atom of ^{18}O. The fact that a similar level of incorporation of ^{18}O into acetate is found in the cleavage of the side-chain of pregnenolone when two pathways operate leading to the formation of **3** and **4** (in the ratio of 1:2–3) indicates that the cleavages in both cases occur by mechanisms that are characterised by the introduction, somehow, of an atom from O_2 into the released acetic acid.

Complementary experiments were performed under $^{16}O_2$ with deuteriated substrates containing ^{18}O in the C-20 carbonyl oxygen (Table 1). By comparing the intensities of molecular ion peaks due to $C^2H_3COOBzl$ and $C^2H_3C^{18}OOBzl$ it was established that 66–87% of C-20 carbonyl oxygen atoms of both the substrates were retained in the released acetic acid.



Scheme 3

Conclusions and Mechanistic Considerations

The main finding of the work is that in the lyase-catalysed conversion of 17α-hydroxypregnenolone into **3**, one atom of oxygen from O_2 is incorporated into the released acetate. A similar incorporation of oxygen into acetate occurs when pregnenolone is converted into the Δ^{16}-steroid **4** and dehydroisoandrosterone **3**. Cumulatively, these results support our earlier suggestion[1,3,4,10] that the two reactions for the cleavage of the side-chain of pregnenolone (**1**→**4**) and 17α-hydroxypregnenolone (**2**→**3**) are examples of a general process conforming to the stoichiometry of Scheme 2 and that these conversions may be rationalised by at least two broad mechanisms. These use either an iron-peroxy or an iron-oxo intermediate known to be formed in the P-450 dependent enzymic reaction.

In Mechanism 1 using the peroxy species the crucial primary event is its reaction with the carbonyl group to produce an adduct **5** (Scheme 3) that decomposes with the incorporation of one atom of oxygen from O_2 into the released acid, RCO_2H. In principle, the fragmentation of the adduct **5** can occur by a heterolytic, homolytic or a pericyclic pathway. The choice between these processes is dictated by geometrical considerations together with the acidity of the X–H bond. In the conversion of 17α-hydroxypregnenolone **2** into dehydroisoandrosterone, the adduct of the type **5** is likely to decompose by an ionic mechanism because of the presence at the X–H position of a readily ionisable OH bond. However, in the formation of the Δ^{16}-steroid since the pK_a of the C-16 carbon acid is expected to be high (> 40) it is advantageous to use a homolytic process (Mechanism 1) in which the adduct **5** is initially converted into ferroxy and alkoxy radicals **6** and then the formation of the products from the latter, **6**, occurs by predictable reactions involving fragmentation and disproportionation. Mechanism 2 uses the iron-oxo intermediate, in the $Fe^{IV}O^{\bullet}$ form, which is implicated in the normal hydroxylation process.[4] In this case the initial reaction is a hydrogen abstraction to give the radical species **7**, which furnishes the products as shown in Scheme 3. The relative merits of these two options and our preference for the mechanism(s) involving an adduct of the type **6** are debated in a forthcoming paper.

Experimental

Chemicals were generally obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset or Sigma Chemical Co., Poole, Dorset. $^{18}O_2$ (97%)–Ar-gas mixture (ratio 1:2) was obtained from Isogas Ltd., Croydon, Surrey. $^2H_2$ (99.5 atom % $^2H_2$) was obtained from Cambrian Gases, Croydon, Surrey. [7-^{3}H]Pregnenolone was obtained from New England Nuclear Research Products, Dupont (UK) Limited, Stevenage, Hertfordshire and ^3H_2O from Amersham International, Little Chalfont, Buckinghamshire.

Gas Chromatography–Mass Spectrometry (GC–MS).—GC–MS was performed on a Hewlett-Packard 5890/VG TS-250 using a 30 m × 0.32 mm i.d. column of DB17 with splitless injection using the conditions given below. The distribution of isotopes in acetate was measured by comparison of the normalised ion signal areas determined by selected ion recording (SIR).

Analysis of Isotopically Labelled Compounds.—All the chemically synthesised isotopically labelled compounds had physical characteristics (NMR, IR, UV, mass spectra, R_F values) consistent with the assigned structures when compared with unlabelled authentic material. Isotopic distributions in the labelled pregnenolone and 17α-hydroxypregnenolone substrates were measured by direct introduction probe MS analysis of the underivatised solids (since some deuterium exchange from labile positions was observed on GC). All mass spectra were recorded in the electron-impact positive-ion mode.

Preparative TLC plates (1 mm thickness) were prepared from silica gel PF_{254} (Merck Ltd., Poole, Dorset), according to the manufacturers instructions. Radio-chromatogram scanning was performed with a Berthold LB 2723 scanner.

[17α,21-$^2H_4$]*Pregnenolone.*[12]—This compound was prepared by dissolving pregnenolone (100 mg) in a mixture of CH_3O^2H (3 cm^3), dry tetrahydrofuran (4 cm^3) and a KO^2H solution [KO^2H (300 mg) dissolved in ^2H_2O (0.3 cm^3)]. After being left at room temp. under Ar for 48 h the solution was neutralised with glacial acetic acid and the product crystallised by slow addition of water. This gave the desired product (60 mg). Analysis by GC–MS showed contamination with isopregnenolone was < 0.4% and had *m/z* (rel. intensity): 318 (7), 319 (16), 320 (100), 321 (29) and 322 (7). The intensities of ions due to D_0 and D_1 isotopomers (*m/z* 316 and 317) were below the threshold for detection.

[21-$^2H_3$]-17α-*Hydroxypregnenolone.*—This compound was prepared by the same method as [17α,21-$^2H_4$]pregnenolone and had *m/z* (rel. intensity): 334 (10), 335 (100), 336 (27) and 337 (6). The intensities of ions due to D_0 and D_1 isotopomers (*m/z* 332 and 333) were below the threshold for detection.

[20-^{18}O;16α,17α,21-$^2H_5$]*Pregnenolone.*—Deuterium was introduced into the 16α, 17α and 21 positions essentially by the method previously described by Marker[13] but using $^2H_2$, followed by base-catalysed exchange.[12] 5% Palladium on

barium sulfate (200 mg) was suspended in freshly distilled tetrahydrofuran (10 cm^3) and pre-reduced under deuterium gas (1 atm). A solution of 3β-hydroxypregna-5,16-dien-20-one acetate (1 g) in freshly distilled tetrahydrofuran (10 cm^3) was added to the mixture which was maintained under deuterium gas (1 atm) with rapid stirring until 1 equiv. of deuterium had been taken up (*ca.* 2 h). The reaction mixture was then diluted with ethyl acetate and the catalyst removed by filtration through Celite. After the filtrate had been evaporated under reduced pressure, the product was crystallised from ethyl acetate–light petroleum to yield a white crystalline solid (0.75 g). This was subjected to exchange with ^2H_2O as described for the synthesis of [17α,21-$^2H_4$]pregnenolone (see above) to give [16α,17α,21-$^2H_5$]pregnenolone which had *m/z* (rel. intensity): 317 (1), 318 (1), 319 (4), 320 (12) and 321 (100). The intensity of the ion due to D_0 (*m/z* 316) was below the threshold of detection. The sample (30 mg) was dissolved in dry tetrahydrofuran (1 cm^3) containing ^2HCl (37% w/v; 0.4 mm^3) to which was added $^2H_2{}^{18}O$ (0.1 cm^3, 97 atom % ^{18}O; 95 atom % ^2H). The vial was tightly stoppered and left at room temp. for 3 days, after which it was poured into sat. aqueous $NaHCO_3$ and extracted into dichloromethane. The extract was washed with water, dried (Na_2SO_4) and evaporated under reduced pressure. The product was recrystallised from ethyl acetate–light petroleum to give the product (20 mg). After a correction for ^{13}C natural abundance had been made, the mass spectrometric analysis gave the following *m/z* (italics), [composition] and (%) distribution: *319* [D_3 plus D, ^{18}O] (1%), *320* [D_2; ^{18}O plus D_4] (9%), *321* [D_3; ^{18}O plus D_5] (18%), *322* [D_4, ^{18}O] (34%) and *323* [D_5; ^{18}O] (31%). The analysis does not provide precise information on the regiospecificity of the two isotopes for all the species but the synthetic methodology dictates that the *m/z* *322* and *323* isotopomers contain ^{18}O. We can, therefore, set the minimum value for the presence of ^{18}O at C-20 which was *ca.* 65%.

The regio- and stereo-selectivity of the deuterium labelling in the pregnenolone substrates was determined by NMR spectrometry using a Varian VXR 500 500 MHz spectrometer. The signals from the 21, 17α and 16α protons were assigned using homonuclear correlation spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY) of the unlabelled steroid. The absence of these signals and the simplification of coupling in neighbouring protons in the one-dimensional H spectra of the deuteriated compounds was used to confirm the position of the deuterium substitution. The residual signals from C-16α, 17α and 21 protons in the deuteriated compound and the signals in the deuterium NMR spectra were used to quantify the deuterium substitution at each position. This showed that for [16α,17α, 21-$^2H_5$]pregnenolone, all the positions were substituted to >95%. In [20-^{18}O;16α,17α,21-$^2H_5$]pregnenolone, the level of substitution was similar except for 17α which was approximately 66% substituted with deuterium.

[20-^{18}O; 17-$^2H_3$]-17α-*Hydroxypregnenolone*.—This compound was prepared by exchanging [21-$^2H_3$]-17α-hydroxypregnenolone with $^2H_2{}^{18}O$ as described above for the synthesis of [20-^{18}O;16α,17α,21-$^2H_5$]pregnenolone. Following correction for ^{13}C natural abundance, the mass spectrometric analysis gave the following *m/z* (italics), [composition] and (%) distribution: *335* [D_3 plus D; ^{18}O] (12%), *336* [D_2; ^{18}O] (30%) and *337* [D_3; ^{18}O] (53%). Thus, the minimum value for ^{18}O labelling at C-20 was 83%.

[17α,21-^{3}H]Pregnenolone and [21-^{3}H]-17α-hydroxypregnenolone. These tritiated steroids were prepared by the methods described for the synthesis of the corresponding deuterium labelled compounds except that ^3H_2O (1 Ci; 0.2 cm^3) and unlabelled methanolic KOH were substituted for the deuterium-containing reagents.

Preparation of a Microsomal Preparation from Pig Testes and Assay of 17α-*Hydroxylase*-17,20-*lyase Activity*.—Fat-free neonatal pig testes (116 g) were homogenised in Tris-HCl buffer (195 cm^3 containing 10 mmol dm^{-3} Tris, 10 mmol dm^{-3} EDTA and 150 mmol dm^{-3} KCl; pH 7.4) using a blender. The homogenate was centrifuged at 10 000 *g* for 30 min. The supernatant was spun at 105 000 *g* for 1 h. The resulting pellets were resuspended in the buffer (35 cm^3) by homogenisation and stored in 1 cm^3 aliquots at −70 °C. A solution of NADP (4 mg), glucose-6-phosphate (9 mg), glucose-6-phosphate dehydrogenase (5 Units) and [17α,21-^{3}H]pregnenolone (0.05 μmol, 0.8 μCi) in dimethylformamide (50 mm^3) was incubated in buffer (100 mmol dm^{-3} potassium phosphate, pH 7.25; 4 cm^3) at 37 °C for 10 min. The enzyme reaction was initiated by adding a suspension of the microsomal fraction (0.1–0.3 mg of microsomal protein in 100 mmol dm^{-3} potassium phosphate buffer pH 7.25 containing 1 mmol dm^{-3} EDTA, 1 cm^3). Samples (0.5 cm^3) were removed and added to dichloromethane (0.5 cm^3) at time intervals of 0, 5, 10, 15, 20 and 25 min. The mixtures were immediately shaken and then centrifuged. The organic layer was discarded and further dichloromethane (0.5 cm^3) was added and the above procedure was repeated. To the resulting aqueous phase was added charcoal, the suspension shaken, left at 4 °C for *ca.* 1 h and finally centrifuged to remove the charcoal. The radioactivity of the aqueous layer was measured by liquid scintillation counting. Typically, the microsomal fraction catalysed the cleavage of 0.5–0.8 nmol of pregnenolone min^{-1} mg^{-1} of protein.

Incubation of Pregnenolone with Testicular Microsomal Fraction under an Atmosphere of Air and the Mass Spectrometric Analysis of the Side-chain as Benzyl Acetate.—A mixture of NADP (5 mg), glucose-6-phosphate (10 mg), glucose-6-phosphate dehydrogenase (10 Units) in 100 mmol dm^{-3} potassium phosphate buffer (6 cm^3; pH 7.25, 1 mmol dm^{-3} in EDTA) and pig testes microsomes (100 mg microsomal protein; 4 cm^3) was rocked at 37 °C for 10–20 min, after which a solution of [16α,17α,21-$^2H_5$]pregnenolone or [20-^{18}O;16α,17α,21-$^2H_5$]-pregnenolone (1 mg) containing [17α,21-^{3}H]pregnenolone (0.8 μCi; 16 μCi $μmol^{-1}$) in DMF (50 mm^3) was added with swirling. The incubation mixture was shaken at 37 °C for 75 min and following acidification (5% phosphoric acid; 2 cm^3) was freeze-dried. The distillate was then basified with 0.4 mol dm^{-3} aqueous sodium hydroxide (100 mm^3) and again freeze-dried. The residue, after dissolution in water (3 × 200 mm^3), was transferred to a small vial. This solution was freeze-dried and the contents of the vial were dissolved in methanol (80 mm^3) and the solution acidified to pH 1–2 with 1 mol dm^{-3} hydrochloric acid in tetrahydrofuran–water (9:1; 35 mm^3). Diazotoluene in tetrahydrofuran was then immediately added to the preceding solution in aliquots until a permanent red colour persisted. The resulting benzyl acetate was analysed using the following GC–MS conditions: injector 220 °C, initial column temperature 90 °C for 3 min, programmed 4 °C min^{-1} to 140 °C for 1 min, then 25 °C min^{-1} to 220 °C for 5 min, sample transfer lines 240 °C. Sample sizes were in the range 1 mm^3 to 2 mm^3 and the retention time of benzyl acetate was 9.5 min. SIR traces were taken of the ions of mass 150.1 to 157.1 (M^+ = 150.1) and the full scans of range *m/z* 180 to 80, resolution 500. In some experiments with pregnenolone as substrates, a tracer amount of [7-^{3}H]pregnenolone (2 μCi; 25 Ci $μmol^{-1}$) was added to allow the steroid products to be analysed. This was done by extraction with ethyl acetate–methanol (9:1; 3 × 15 cm^3) followed by separation on preparative TLC plates developed in chloroform–ethyl acetate (4:1). The steroids were located by radiochromatogram scanning and eluted from the silica gel with ethyl acetate–methanol (9:1). The steroids were identified and quantified by GC–MS. The

relative ratios of the steroid products were determined from their peak areas, in the total ion chromatograms. This gave a ratio of 3β-hydroxypregna-5,16-diene to dehydroisoandrosterone of 2–3:1.

Incubations under $^{18}O_2$ *Gas.*—The procedure was similar to that described, with the following modifications. The vessel containing the NADPH-generating system and the microsomes underwent three rounds of deaeration under vacuum (water pump) and flushing with argon and were subsequently left at 37 °C for 15 min (to allow the reduction of $NADP^+$). The appropriate amounts of one of the ^2H-labelled pregnenolone admixed with tritiated pregnenolone were added, followed immediately by three cycles of evacuation under reduced pressure and flushing with argon. A 1:2 mixture of $^{18}O_2$ (99.5%)–argon was introduced into the vessel and the tap closed. The incubation mixture was shaken at 37 °C for 75 min and the enzymic products were isolated as for the experiment conducted under unlabelled oxygen.

Incubations with 17α-*Hydroxypregnenolone.*—The incubations with 17α-hydroxypregnenolone were performed in a manner identical with that for pregnenolone, using the appropriately labelled species (1 mg) admixed with [21-^3H]-17α-hydroxypregnenolone (1.3 μCi; 26 μCi μmol^{-1}).

Acknowledgements

We thank the SERC for a grant in support of this work, Miss Ann Roberts for valuable technical assistance, and Dr. D. Turner and Dr. D. Rees (Chemistry Department, University of Southampton) for 500 MHz NMR spectra. This is a contribution from the SERC Molecular Recognition Centre, Southampton University.

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Paper 3/05624D
Received 17*th September* 1993
Accepted 15*th October* 1993