*Biochem. J.* (1980) **185**, 411–421
*Printed in Great Britain*



# Mechanistic and Stereochemical Studies on 3-Oxo Steroid $\Delta^4$–$\Delta^5$-Isomerase from Human Placenta

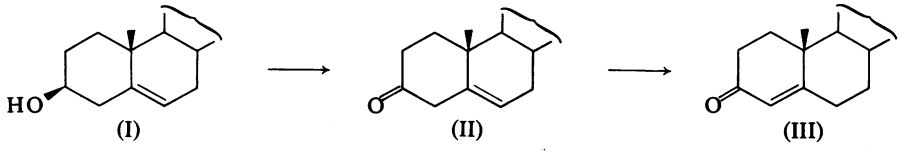
Muhammad AKHTAR, Michael CALDER, Trevor SMITH and James N. WRIGHT
*Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.*

(*Received* 20 *July* 1979)

The mechanism of isomerization of $\Delta^5$-3-oxo steroids to $\Delta^4$-3-oxo steroids was examined by using the membrane-bound 3-oxo steroid $\Delta^4$–$\Delta^5$-isomerase (EC 5.3.3.1) and the 3$\beta$-hydroxy steroid dehydrogenase present in the microsomal fraction obtained from full-term human placenta. (1) Methods for the preparation of androst-5-ene-3$\beta$,17$\beta$-diol specifically labelled at the 4$\alpha$-, 4$\beta$- or 6-positions are described. (2) Incubations with androst-5-ene-3$\beta$,17$\beta$-diol stereospecifically $^3$H-labelled either in the 4$\alpha$- or 4$\beta$-position showed that the isomerization reaction occurs via a stereospecific elimination of the 4$\beta$ hydrogen atom. In addition, the complete retention of $^3$H in the $\Delta^4$-3-oxo steroids obtained from [4$\alpha$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol indicates that the non-enzymic contribution to these experiments was negligible. (3) To study the stereochemistry of the insertion of the incoming proton at C-6, the [6-$^3$H]androst-4-ene-3,17-dione obtained from the oxidation isomerization of [6-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol was enzymically hydroxylated in the 6$\beta$-position by the fungus *Rhizopus stolonifer*. Retention of $^3$H in the 6$\alpha$-position of the isolated 6$\beta$-hydroxyandrost-4-ene-3,17-dione indicates that in the isomerase-catalysed migration of the $C_{(5)}$=$C_{(6)}$ double bond, the incoming proton from the acidic group on the enzyme must enter C-6 from the $\beta$-face, forcing the existing $^3$H into the 6$\alpha$-position.

The conjugated ketone system present in the A-ring of several groups of steroid hormones is formed from the corresponding $\Delta^5$-3$\beta$-hydroxy precursors (structure of the type I in Scheme 1) through the participation of two enzymic reactions as shown in the generalized sequence of Scheme 1. The first step in the sequence is a nicotinamide nucleotide-linked oxidation of the 3$\beta$-hydroxy group to give the $\beta,\gamma$-unconjugated-ketone system (II, Scheme 1), which is then converted into the conjugated ketone (III, Scheme 1) by an isomerase. Our present-day knowledge of the mechanism of the isomerization reaction has its origin in a rather bizarre observation made during the middle 1950s, when it was found that a soil bacterium, *Pseudomonas testosteroni*, could utilize testosterone as the sole carbon source for its growth and also possessed a powerful activity for the isomerization of $C_{19}$ $\Delta^5$-3-oxo steroids (II) into the corresponding $\Delta^4$-3-oxo isomers (III) (Talalay *et al.*, 1952; Talalay & Wang, 1955; Talalay & Benson, 1972). Since this original discovery, several aspects of this enzyme have been studied and, in particular, the classical work of Talalay and his colleagues has shown that the isomerization reaction involves an intramolecular migration of the 4$\beta$ hydrogen atom to the 6$\beta$-position (for a review, see Talalay & Benson, 1972).

In contrast with the wealth of information available on the bacterial enzyme, progress in determining the mechanism of isomerization by its mammalian counterparts has been less dramatic, owing to the low activity of the enzyme in the endocrine tissues used so far, and the consequent difficulty of assessing the size of the non-enzymic contribution to the isomerization [this aspect is critically discussed by Talalay & Benson (1972)]. We have, however,




(I) (II) (III)

Scheme 1. *Pathway for the conversion of* $\Delta^5$-3$\beta$-*hydroxy steroids into* $\Delta^4$-3-*oxo steroids*



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shown (Skinner & Akhtar, 1968) that a microsomal fraction from full-term human placenta possesses an adequate activity for the conversion of $\Delta^5$-3$\beta$-hydroxyandrostane derivatives of type (I) into the corresponding conjugated ketones (III).

The mechanism of the isomerization process (II→III) can therefore in principle be studied by using a suitably labelled $\Delta^5$-3$\beta$-hydroxy steroid (I), where the $\Delta^5$-3-oxo steroid (II) produced by the dehydrogenase is utilized by the isomerase. An account of such a study is described below.

## Experimental

### *Materials*

Chemicals were generally obtained from BDH, Poole, Dorset, U.K., except for special chemicals obtained from the following sources: 3$\beta$-hydroxy-[4-$^{14}$C]androst-5-en-17-one (The Radiochemical Centre, Amersham, Bucks., U.K.); 3$\beta$-hydroxyandrost-5-en-17-one (Searle, Morpeth, Northd., U.K.). A freeze-dried culture of the honey fungus *Rhizopus stolonifer* was generously given by the Commonwealth Mycological Institute, Kew, Surrey, U.K.

### *Measurement of radioactivity and t.l.c.*

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

### *Preparation of placental microsomal fractions*

Human term placentas were obtained within 1 h of birth and kept at 4°C. The following procedure, based on the original method of Ryan (1959), was also carried out at 4°C.

The cotyledon tissue was severed from the chorionic plate, cut into small pieces, added to 50mM-sodium phosphate buffer, pH 7.4, containing 0.25M-sucrose [buffer/tissue, 1:2 (v/w)] and homogenized in an Atomix for five 30s intervals, with 2min cooling periods. The homogenate was centrifuged at 10000*g* for 10min, the supernatant was decanted off and centrifuged for 60min at 105000*g*. The microsomal pellet was resuspended in 50mM-sodium phosphate buffer, pH 7.4, dispersed with a hand homogenizer and then re-centrifuged for 60min at 105000*g*. The resultant supernatant was decanted off and the washed microsomal pellet resuspended in 50mM-sodium phosphate buffer, pH 7.4 (50mg of protein/ml of buffer) and stored at −20°C in 2.5ml portions.

### *Microsomal incubations*

[4-$^{14}$C,4$\beta$-$^3$H]Androst-5-ene-3$\beta$,17$\beta$-diol (200$\mu$g; $^3$H/$^{14}$C = 10.36; 25000d.p.m. of $^{14}$C) in methanol (25$\mu$l) was added to 2.5ml of the microsomal suspension above containing $NAD^+$ (5mg). The incubations were carried out at 37°C with shaking. 17$\beta$-Hydroxyandrost-4-en-3-one (100$\mu$g in 20$\mu$l of methanol) was added to the mixture after 20min to saturate the 17$\beta$-hydroxy steroid dehydrogenase with non-radioactive substrate and the incubation continued for another 10min. Steroid products were extracted with ethyl acetate (3 × 10ml). The extracts were pooled, dried over anhydrous $Na_2SO_4$ and solvent removed under reduced pressure. The residue containing 20000d.p.m. of $^{14}$C (80% recovery) was applied to preparative silica-gel $PF_{254}$ plates, which were developed in dichloromethane/acetone (4:1, v/v). The bands corresponding to authentic samples of androst-5-ene-3$\beta$,17$\beta$-diol ($R_F$ 0.33), 3$\beta$-hydroxyandrost-5-en-17-one ($R_F$ 0.52) 17$\beta$-hydroxyandrost-4-en-3-one ($R_F$ 0.41) and androst-4-ene-3,17-dione ($R_F$ 0.62) were eluted with ethyl acetate. The ethyl acetate fractions were evaporated to dryness and each residue was admixed with appropriate non-radioactive carrier (20mg) and the solid recrystallized. The results are shown in Table 1.

Incubations with [4-$^{14}$C,4$\alpha$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol (400$\mu$g; $^3$H/$^{14}$C = 6.15; 40000d.p.m. of $^{14}$C) in methanol (25$\mu$l) were carried out under conditions similar to those described for [4-$^{14}$C,4$\beta$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol except for the following changes. The incubations were carried out for 1h in the presence of $NAD^+$ (10mg), and 17$\beta$-hydroxyandrost-4-en-3-one (200$\mu$g in 20$\mu$l of methanol) was added after 45min. The total recovery of $^{14}$C radioactivity was about 90% and the results are shown in Table 1.

The $^3$H in androst-4-ene-3,17-dione derived from the [4$\alpha$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol was shown to be still resident at C-4 by the following experiment. The biosynthetic androst-4-ene-3,17-dione was admixed with carrier to give a sample (25mg; $^3$H/$^{14}$C = 6.24; 10000d.p.m. of $^3$H/mg) that was dissolved in acetic acid (2.5ml) and in the presence of 10% (w/w) Pd on charcoal (10mg) hydrogenated at room temperature and at normal atmospheric pressure (101325Pa) for 5h. The resulting mixture was filtered, and the filtrate evaporated to dryness under reduced pressure. The residue was applied to preparative-t.l.c. silica-gel $PF_{254}$ plates, which were developed in cyclohexane/ethyl acetate (2:1, v/v). The band ($R_F$ 0.35) containing the isomeric mixture of 5$\alpha$- and 5$\beta$-androstane-3,17-dione (Nishimura & Shimahara, 1966) was eluted with ethyl acetate. The solvent was removed under reduced pressure, an oily residue being left. The i.r. spectrum of the residue showed the absence of a band at 1660cm$^{-1}$ (conjugated ketone) and the appearance of a band at 1695cm$^{-1}$ (unconjugated ketone). The $^3$H/$^{14}$C ratio of the product was 5.85. The oily residue was dissolved in methanolic 5% (w/v) KOH (5ml), the mixture flushed with $N_2$ and sealed in a glass vial. This was allowed to equilibrate for 24h at room tempera-

Table 1. *Stereospecificity of hydrogen elimination from C-4 during the isomerase-catalysed reaction*

The doubly labelled samples of [4-$^{14}$C,4$\beta$-$^3$H]- and [4-$^{14}$C,4$\alpha$-$^3$H]-androst-5-ene-3$\beta$,17$\beta$-diol ($^3$H/$^{14}$C ratio 10.36 and 6.15 respectively) were incubated with placental microsomal fractions as described in the Experimental section. The steroid products were extracted with ethyl acetate and separated by t.l.c.; the bands corresponding to authentic references were extracted and co-crystallized to constant specific activity. The Roman numerals below refer to the structures in Scheme 5.

| | $^{14}$C radioactivity (d.p.m.) | $^3$H/$^{14}$C ratio | $^3$H loss (%) |
|---|---|---|---|
| (*a*) Incubation with the 4$\beta$-$^3$H-labelled substrate | | | |
| Androst-5-ene-3$\beta$,17$\beta$-diol (VII; R = -H, -OH) (recovered) | 3667 (17.8)* | 10.36 | — |
| 3$\beta$-Hydroxyandrost-5-en-17-one (VII; R = O) | 2490 (12.1) | 10.05 | 3 |
| 17$\beta$-Hydroxyandrost-4-en-3-one (XIV; R = -H, OH) | 5309 (25.8) | 1.02 | 90.2 |
| Androst-4-ene-3,17-dione (XIV; R = O) | 9180 (44.3) | 0.87 | 91.7 |
| (*b*) Incubation with the 4$\alpha$-$^3$H-labelled substrate | | | $^3$H increase (%) |
| Androst-5-ene-3$\beta$,17$\beta$-diol (VII; R = -H, -OH) (recovered) | 14038 (39) | 6.13 | — |
| 3$\beta$-Hydroxyandrost-5-en-17-one (VII; R = O) | 6363 (17.6) | 6.17 | 1 |
| 17$\beta$-Hydroxyandrost-4-en-3-one (XIV; R = -H, OH) | 6069 (16.8) | 6.21 | 1.3 |
| Androst-4-ene-3,17-dione (XIV; R = O) | 9537 (26.5) | 6.24 | 1.8 |

* The values in parentheses represent the percentages of $^{14}$C radioactivity present in each compound relative to the total extracted.

ture and the product was extracted with ethyl acetate (3 × 10ml) and purified as described above. The $^3$H/$^{14}$C ratio of the isomeric mixture of 5$\alpha$- and 5$\beta$-androstane-3,17-dione was now 0.61.

*Hydroxylation of androst-4-ene-3,17-dione by Rhizopus stolonifer*

[4-$^{14}$C,6-$^3$H]Androst-4-ene-3,17-dione ($^3$H/$^{14}$C = 18.6) was obtained by microsomal-oxidation/isomerization of [4-$^{14}$C,6-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol ($^3$H/$^{14}$C = 19.1) as described above and subjected to hydroxylation essentially by the method of Eppstein *et al.* (1952) as detailed below.

Cultures of *Rhizopus stolonifer* 57762 were maintained on agar slopes [Oxoid agar no. 3 (1.2%, w/v)/Oxoid malt extract (5%, w/v)]. Slopes were incubated at 30°C for 2 days to produce a vigorous growth of white mycelia and conidia. These were stored at 4°C until required.

Mycelia and conidia were transferred from stock slopes to 5ml of broth [Bacteriological Peptone (Oxoid no. L34; 2%, w/v), Oxoid malt extract (5%, w/v) and glucose (5%, w/v) in tap water]. These were incubated at room temperature until a vigorous mycelial growth occurred (approx. 2 days). A portion of this culture (1 ml) was then inoculated into 20ml broth cultures in 100ml conical flasks and incubated for 2 days.

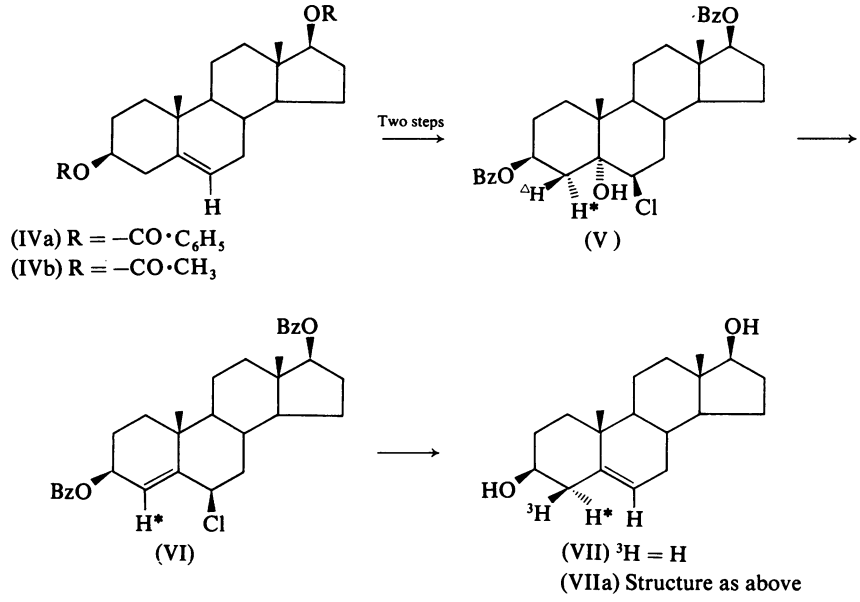
[4-$^{14}$C,6-$^3$H]Androst-4-ene-3,17-dione (sp. radioactivities 2500000d.p.m. of $^3$H and 133000d.p.m. of $^{14}$C/mg) (3mg in 30$\mu$l of methanol) was added to each flask, and the incubation continued for a further 24h.

Steroid products were extracted from mycelia into dichloromethane by repeated rotary mixing and centrifugation. Approx. 60% of initially incubated $^{14}$C radioactivity was recovered in this way. The combined dichloromethane extracts were washed with $NaHCO_3$ and water, then dried over anhydrous $Na_2SO_4$, and the solvent removed under reduced pressure. The residue was applied to preparative silica-gel $PF_{254}$ plates, which were developed in chloroform/methanol (50:1, v/v). The bands corresponding to authentic androst-4-ene-3,17-dione ($R_F$ 0.66) and 6$\beta$-hydroxyandrost-4-ene-3,17-dione ($R_F$ 0.30) were extracted with ethyl acetate/methanol (9:1, v/v) and co-crystallized with non-radioactive material to constant specific radioactivity. The unconverted substrate, androst-4-ene-3,17-dione, and the product, 6$\beta$-hydroxyandrost-4-ene-3,17-dione, contained 15 and 20% respectively of the recovered $^{14}$C radioactivity. A further major band ($R_F$ 0.25) containing 55% of the recovered $^{14}$C radioactivity was removed and subjected to purification in a second solvent system, benzene/ethyl acetate (10:1, v/v). A single band with $R_F$ 0.45 was observed, which had a $^3$H/$^{14}$C ratio of 20.28, and corresponded to the material previously characterized as 11$\alpha$-hydroxyandrost-4-ene-3,17-dione (Eppstein *et al.*, 1954). The $^3$H/$^{14}$C ratios for the above-mentioned samples are shown in Table 2 below.

*Synthesis of [4$\beta$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol (VIIa, Scheme 2)*

The starting material for this synthesis was 6$\beta$-chloroandrost-4-ene-3$\beta$,17$\beta$-diol dibenzoate (VI), which was synthesized via the 5,6$\alpha$-oxide as described by Malhotra & Ringold (1965).

A solution of 6$\beta$-chloroandrost-4-ene-3$\beta$,17$\beta$-diol dibenzoate (VI) (50mg) in dry diethyl ether (10ml) was treated with $LiAl^3H_4$ (5mCi; 15mg) and the reaction mixture was refluxed for 2h. Excess $LiAlH_4$ (20mg) was added and the mixture again refluxed for further 2h. The reaction mixture was cooled to 0°C and ethyl acetate (2.5ml) was slowly added, followed by acidification to pH 1.0 with 0.2M-HCl. The product was extracted with ethyl acetate (3 × 10ml) and the combined extracts were washed




Scheme 2. *Sequence of reactions for the synthesis of* [4β-$^3$H]*androst-5-ene-3β,17β-diol*

with saturated $NaHCO_3$ (3 × 10 ml) and then with water (3 × 10 ml). After the extract was dried over anhydrous $Na_2SO_4$, the solvent was removed under reduced pressure. The residue was applied to preparative silica-gel $PF_{254}$ plates, which were developed in chloroform/methanol (100:2, v/v), and the band corresponding to authentic androst-5-ene-3β,17β-diol was eluted with ethyl acetate. The solvent was removed under reduced pressure and the residue crystallized from acetone/hexane giving [4β-$^3$H]androst-5-ene-3β,17β-diol (VIIa; yield 22 mg; m.p. 179–181°C; 1 250 000 d.p.m. of $^3$H/mg).

A doubly labelled sample of [4-$^{14}$C,4β-$^3$H]-androst-5-ene-3β,17β-diol was obtained by admixing [4-$^{14}$C]androst-5-ene-3β,17β-diol (produced from $NaBH_4$ reduction of commercially available 3β-hydroxy[4-$^{14}$C]androst-5-en-17-one) and the 4β-$^3$H-labelled diol (VIIa).

*Confirmation of the location of* $^3$*H in* [4β-$^3$*H*]-*androst-5-ene-3β,17β-diol*

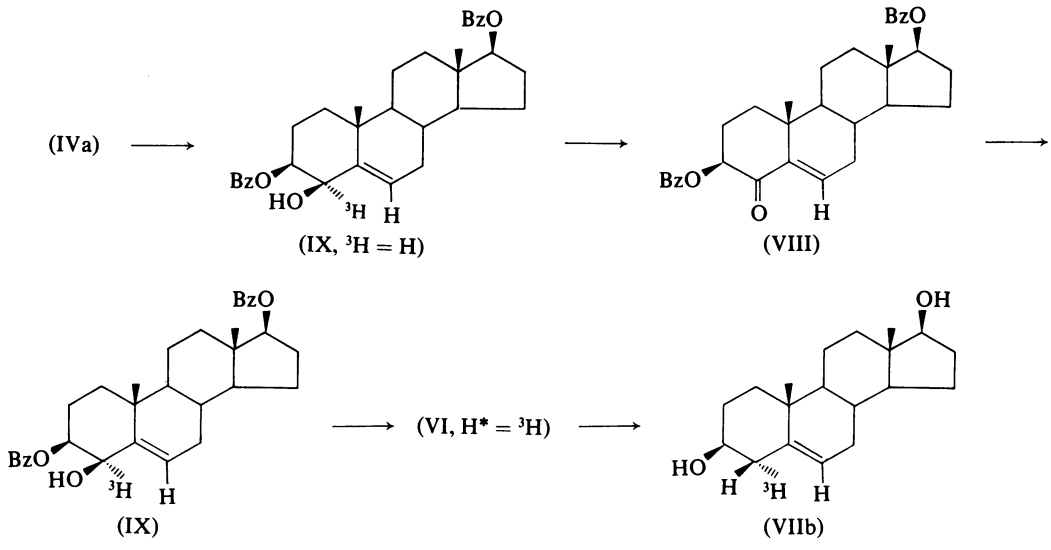
By using the method of Malhotra & Ringold (1965), a doubly labelled sample of [4-$^{14}$C,4β-$^3$H]-androst-5-ene-3β,17β-diol having a $^3$H/$^{14}$C ratio of 5.02 was converted into 6β-chloroandrostane-3β,5α,17β-triol 3,17-dibenzoate (V, $H^{\Delta} = {}^3H$; $^3$H/$^{14}$C = 4.87); m.p. 223–225°C; $\nu_{max.}$ (Nujol) 3450 $cm^{-1}$.

Dehydration of the latter compound with $POCl_3$ gave 6β-chloroandrost-4-ene-3β,17β-diol dibenzoate (VI; $^3$H/$^{14}$C = 0.55); m.p. and mixed m.p. with authentic 6β-chloroandrost-4-ene-3β,17β-diol dibenzoate, 166–168°C.

*Synthesis of* [4α-$^3$*H*]*androst-5-ene-3β,17β-diol* (*VIIb, Scheme* 3)

*Androst-5-ene-3β,4β,17β-triol* 3,17-*dibenzoate* (*IX, Scheme* 3; $^3H = H$). $SeO_2$ (1.2 g) in acetic acid/water (100:1, v/v; 50 ml) was added to a solution of androst-5-ene-3β,17β-diol dibenzoate (IVa) (5.0 g) in acetic acid (150 ml) and the reaction mixture refluxed for 6 min. Sodium acetate (8.0 g) was added to the reaction flask and the solution filtered. The precipitate obtained by the addition of water to the cooled filtrate was filtered, redissolved in dichloromethane (25 ml) and successively washed with saturated $NaHCO_3$ (3 × 25 ml) and then with water (3 × 25 ml). After the extract was dried over anhydrous $Na_2SO_4$ the solvent was removed under reduced pressure. Recrystallization of the product from dichloromethane/light petroleum (b.p. 60–80°C) gave androst-5-ene-3β,4β,17β-triol 3,17-dibenzoate (1.3 g); m.p. 224–225°C; analytical t.l.c. on silica-gel $GF_{254}$ in benzene/ethyl acetate (4:1, v/v) gave a single spot of $R_F$ 0.65; $\nu_{max.}$ (Nujol) 1610, 1715, 3540 $cm^{-1}$; $^1$H n.m.r. (100 MHz, [$^2$H]-chloroform) δ1.32 (3H, s, 19-$CH_3$), δ5.0 (H, d, 4α-H); *m/e* 514 [$M^+$]; (Found C, 77.12; H, 7.44; $C_{33}H_{38}O_5$ requires C, 77.04; H, 7.40).

4-*Oxoandrost-5-ene-3β,17β-diol* 3,17-*dibenzoate* (*VIII*). Androst-5-ene-3β,4β,17β-triol 3,17-dibenzoate (700 mg) was dissolved in dry pyridine (35 ml) and $CrO_3$ (1.5 g) was slowly added. After 4 h at 50–55°C the reaction mixture was poured into cold water and the product extracted with dichloromethane (3 × 50 ml). The combined extracts were




Scheme 3. *Sequence of reactions for the synthesis of* [4α-$^3$H]*androst-5-ene-3β,17β-diol*

successively washed with 1 M-HCl (3 × 25 ml), saturated $NaHCO_3$ (3 × 25 ml), and water (3 × 25 ml). After drying the extract over anhydrous $Na_2SO_4$ the solvent was removed under reduced pressure. The residual oil was applied to preparative silica-gel $PF_{254}$ plates, which were developed in benzene/ethyl acetate (4 : 1, v/v). The band containing the 4-oxo compound ($R_F$, 0.75) was eluted with ethyl acetate and the solvent removed under reduced pressure. The residue was crystallized from ethyl acetate/light petroleum (b.p. 60–80°C) to give 4-oxoandrost-5-ene-3β,17β-diol dibenzoate (VIII), (120 mg); m.p. 183–184°C; $\nu_{max.}$ (Nujol) 1610, 1715, 1640 $cm^{-1}$; $^1$H n.m.r. (100 MHz [$^2$H]chloroform) $\delta = 1.03$ p.p.m. (3H, s, 19-$CH_3$) loss of 4α-H doublet centred at $\delta = 5.0$ p.p.m.; *m/e* 512 ($M^+$) (Found C, 77.10; H, 7.07; $C_{33}H_{36}O_5$ requires C, 77.04; H, 7.00).

[4α-$^3$H]*Androst-5-ene-3β,4β,17β-triol* 3,17-*dibenzoate* (*IX*). 4-Oxoandrost-5-ene-3β,17β-diol dibenzoate (VIII) (25 mg) was reduced with $NaB^3H_4$ (2 mg; 10 mCi) in tetrahydrofuran/methanol (1 : 1, v/v; 2.5 ml), and the reaction mixture left for 45 min at room temperature. After the addition of $NaBH_4$ (10 mg), the reaction mixture was left for a further 45 min and then diluted with water (10 ml). The mixture was extracted with dichloromethane (3 × 10 ml) and the combined extract washed with water (3 × 10 ml) and dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure and the product recrystallized from dichloromethane/light petroleum (b.p. 60–80°C) to give [4α-$^3$H]androst-5-ene-3β,4β,17β-triol 3,17-dibenzoate (18 mg; 2 900 000 d.p.m. of $^3$H/mg). With respect to m.p., $^1$H n.m.r., i.r. and t.l.c. mobility the compound was shown to be identical with the allylic alcohol (IX; $^3H = H$).

The [4α-$^3$H]androst-5-ene-3β,4β,17β-triol 3,17-dibenzoate (IX) was co-crystallized with 30 mg of non-radioactive compound, to a final specific radioactivity of 1 000 000 d.p.m. of $^3$H/mg.

A 5.0 mg sample of the material was further co-crystallized with 50 mg of non-radioactive compound (90 000 d.p.m. of $^3$H/mg), then oxidized with $CrO_3$ as described above. The specific radioactivity of the product, 4-oxoandrost-5-ene-3β,17β-diol dibenzoate, was $1.9 \times 10^3$ d.p.m. of $^3$H/mg.

*6β-Chloro*[4-$^3$H]*androst-4-ene-3β,17β-diol dibenzoate* (*VI*, $H^* = {}^3H$). Thionyl chloride (0.15 ml) was added to a solution of [4α-$^3$H]androst-5-ene-3β,4β,17β-triol 3,17-dibenzoate (IX) (50 mg), in dry pyridine (5 ml) at 0°C. After 2 min, ice-cold water (30 ml) was added and the mixture was extracted with dichloromethane (3 × 20 ml). The combined extracts were dried over anhydrous $Na_2SO_4$ and the solvent was removed under reduced pressure. The residue was applied to preparative t.l.c. silica-gel $PF_{254}$ plates, which were developed in benzene/ethyl acetate (4 : 1, v/v). The band corresponding to authentic 6β-chloroandrost-4-ene-3β,17β-diol dibenzoate was removed and crystallized from acetone/hexane to yield 18 mg, m.p. 167–169°C. Further analysis of the 6β-chloro compound obtained by the above synthesis showed it to be identical in all respects with the 6β-chloroandrost-4-ene-3β,17β-diol dibenzoate obtained by the dehydration of 6β-chloroandrostane-3β,5α,17β-triol 3,17-dibenzoate.

[4α-$^3$H]*Androst-5-ene-3β,17β-diol* (*VII*). A solution of [4-$^3$H]6β-chloroandrost-4-ene-3β,17β-diol dibenzoate (25 mg) in dry diethyl ether (10 ml) was treated with $LiAlH_4$ (15 mg) and the reaction mixture refluxed for 2 h. The reaction product, [4α-$^3$H]-androst-5-ene-3β,17β-diol was isolated and purified

as described above, yield 9 mg, specific radioactivity 625 000 d.p.m. of $^3H$/mg. A doubly labelled sample, [4-$^{14}C$,4$\alpha$-$^3H$]androst-5-ene-3$\beta$,17$\beta$-diol (625 000 d.p.m. of $^3H$; 100 000 d.p.m. of $^{14}C$/mg) was prepared as described above.

*Synthesis of* [6-$^3H$]*androst-5-ene-3β-diol* (*VIIc*, *Scheme* 4)

The starting material for this synthesis was 5$\alpha$-bromoandrostane-3$\beta$,6$\beta$,17$\beta$-triol 3,17-diacetate, which was prepared by the method of Akhtar & Barton (1964).

*6-Oxoandrostane-3β,17β-diol diacetate* (*XI*). 5$\alpha$-Bromoandrostane-3$\beta$,6$\beta$,17$\beta$-triol 3,17-diacetate (1 g) in acetone (10 ml) was treated with Jones reagent (Bowden *et al.*, 1946; 0.7–0.9 ml) until an orange colour persisted. Chromium (II) salts that precipitated during the reaction were removed by filtration. Slow addition of water to the resulting filtrate gave the crystalline product (X; 700 mg). The latter material was dissolved in acetic acid/water (20:1, v/v) (20 ml) and stirred with zinc dust (6.0 g) at 20°C for 5 h. The zinc was removed by gravity filtration and the filtrate, after the addition of water (100 ml), was extracted with dichloromethane (3 × 30 ml). The combined extracts were washed with saturated $NaHCO_3$ (3 × 25 ml) and water (3 × 25 ml). After drying the extract over anhydrous $Na_2SO_4$ the solvent was removed under reduced pressure. Recrystallization from dichloromethane/light petroleum (b.p. 60–80°C) gave 6-oxoandrostane-3$\beta$,17$\beta$-diol diacetate (XI); yield 400 mg, m.p. 169–173°C.

[6$\alpha$-$^3H$]*Androstane-3β,6β,17β-triol* 3,17-*diacetate* (*XII*). 6-Oxoandrostane-3,17-diol diacetate (XI; 90 mg) in methanol (5 ml) was treated with $NaB^3H_4$ (17 mg; 2.6 mCi) and the reaction mixture left at 20°C for 45 min. Excess $NaBH_4$ was added to the reaction mixture, which was left for 45 min. After the addition of water (10 ml) the resulting crystalline suspension was filtered. Recrystallization from dichloromethane/light petroleum (b.p. 60–80°C) gave [6$\alpha$-$^3H$]androstane-3$\beta$,6$\beta$,17$\beta$-triol 3,17-diacetate; yield 70 mg, m.p. 77–79°C. Analytical t.l.c. on silica-gel $GF_{254}$ plates, developed in chloroform/methanol (50:1, v/v) gave a single band with $R_F$ 0.53.

[6-$^3H$]*Androst-5-ene-3β,17β-diol* (*VIIc*). A mixture of [6$\alpha$-$^3H$]androstane-3$\beta$,6$\beta$,17$\beta$-triol 3,17-diacetate (XII), (60 mg) and $POCl_3$ (0.2 ml) in dry pyridine (0.7 ml) was refluxed for 5 min and left at 20°C for 18 h. After the addition of water (4 ml) the mixture was extracted with diethyl ether (3 × 10 ml) and the combined extracts worked up as described above. The residue was crystallized from ethyl acetate/light petroleum (b.p. 60–80°C) giving [6$^3H$]androst-5-ene-3$\beta$,17$\beta$-diol diacetate; yield 30 mg, m.p. 152–154°C.

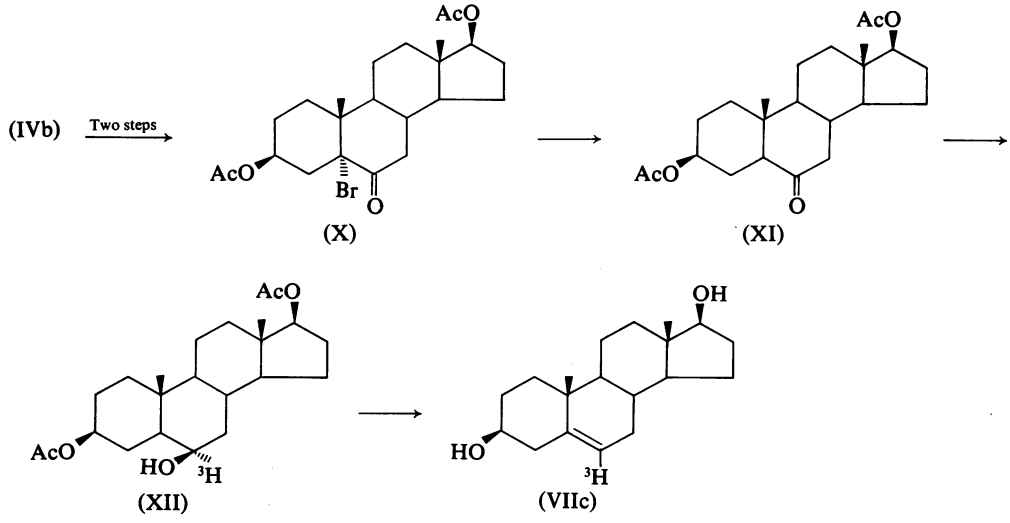
Hydrolysis of the diacetate with methanolic 5% KOH gave [6-$^3H$]androst-5-ene-3$\beta$,17$\beta$-diol; yield 18 mg, m.p. 176–178°C.

A doubly labelled sample, [4-$^{14}C$,6-$^3H$]androst-5-ene-3$\beta$,17$\beta$-diol (2 500 000 d.p.m. of $^3H$; 133 000 d.p.m. of $^{14}C$/mg), was prepared by the method described above.

## Results and Discussion

*Synthesis of* [4$\beta$-$^3H$]- *and* [4$\alpha$-$^3H$]-*androst-5-ene-3β,17β-diol* (*VIIa and VIIb respectively*)

Our projected studies on the mechanism of the isomerization by placental 3-oxo steroid $\Delta^4$–$\Delta^5$-iso-




Scheme 4. *Sequence of reactions for the synthesis of* [6-$^3H$]*androst-5-ene-3β,17β-diol*

merase required the determination of the fate of both the 4$\alpha$ and 4$\beta$ hydrogen atoms of a suitable precursor during its conversion into $\Delta^4$-3-oxo steroid. For this purpose, two stereospecifically (C-4) $^3$H-labelled samples of androst-5-ene-3$\beta$,17$\beta$-diol (VIIa and VIIb) were prepared. [4$\beta$-$^3$H]Androst-5-ene-3$\beta$,17$\beta$-diol (VIIa) was obtained by the method originally developed by Malhotra & Ringold (1965) for the synthesis of the corresponding $^2$H analogue as outlined in Scheme 2, whereas [4$\alpha$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol (VIIb) was prepared by modification of the method used by Achmatowicz *et al.* (1973) in the synthesis of [4$\alpha$-$^2$H]cholesterol as outlined in Scheme 3.

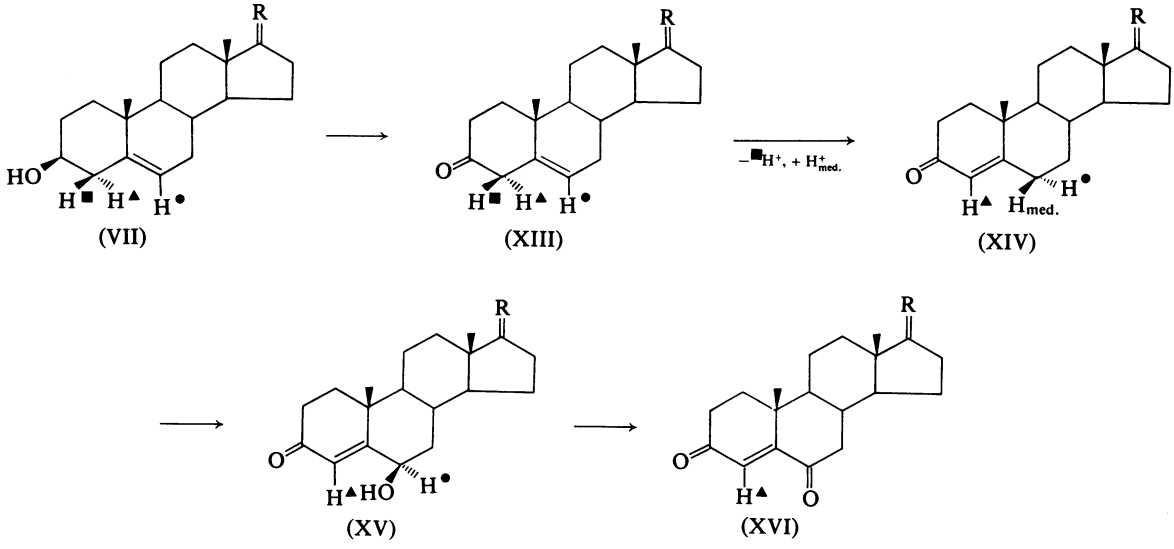
A crucial step in the synthesis of compounds (VIIa) (Scheme 2) and (VIIb) (Scheme 3) is the reductive dehalogenation of a $\Delta^{4,5}$-6-chloro system of type (VI) with $LiAlH_4$. Ireland *et al.* (1958) had previously shown that reduction of 6$\beta$-chlorocholest-4-en-3$\beta$-ol benzoate with $LiAl^2H_4$ resulted in production of a single stereospecifically labelled product, namely [4$\beta$-$^2$H]cholesterol. In the present paper, support for the proposed stereochemical course was obtained by converting compound (VIIa) into the chlorohydrin (V, $^{\Delta}H = {}^3H$) with complete retention of $^3$H. Subsequent dehydration of (V, $^{\Delta}H = {}^3H$) with $POCl_3$, resulted in a >90% loss of $^3$H label. Since the dehydration reaction is proposed to occur via a diaxial-elimination process, the loss of radioactivity indicates that at least 90% of the $^3$H in the chlorohydrin (V, $^{\Delta}H = {}^3H$) and hence in its precursor (VIIa) was located at the 4$\beta$-position. The crucial stage in the preparation of [4$\alpha$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol involved the conversion of the dibenzoate (IVa) into the allylic alcohol (IX; $^3H = H$), which was achieved by the $SeO_2$ method originally introduced by Petrov *et al.* (1943). Confirmation of the 4$\beta$-orientation of the hydroxy group in compound (IX) came from the examination of its $^1$H n.m.r. spectrum, which showed a significant downfield shift ($\delta$) of the C-19 methyl peak ($\delta = 1.33$ p.p.m.) as compared with that found ($\delta = 1.05$ p.p.m.) in the 4-oxo derivative (VIII). This finding is in keeping with the expected magnetic deshielding effect by the 4$\beta$-hydroxy group on the C-19 methyl group (Zürcher, 1963). The allylic alcohol (IX; $^3H = H$) was then oxidized with $CrO_3$/pyridine to furnish the conjugated ketone (VIII), which was smoothly reduced by $NaB^3H_4$ to give the corresponding $^3$H-labelled allylic alcohol (IX). Oxidation of the $^3$H-labelled allylic alcohol (IX) to the parent conjugated ketone (VIII) showed that the $^3$H was located at C-4 to the extent of 98%. The 4$\alpha$-$^3$H labelled allylic alcohol (IX) on treatment with $SOCl_2$ under carefully controlled conditions gave the rearranged chloro compound (VI; $^*H = {}^3H$) which was identical with another sample prepared by the method of Scheme 2. The reductive dechlorination of compound (VI) ($^*H = {}^3H$) with $LiAlH_4$, which would be expected to occur by the delivery of the hydride from the $\beta$-face, forcing the $^3$H into the $\alpha$-orientation, gave the required derivative (VIIb).

Another labelled species of androst-5-ene-3$\beta$,17$\beta$-diol containing $^3$H at C-6 was prepared by the sequence of reactions outlined in Scheme 4.

*Stereochemistry of hydrogen elimination from C-4 during the isomerase-catalysed reaction*

Preliminary experiments established that the incubation of [4-$^{14}$C]androst-5-ene-3$\beta$,17$\beta$-diol (VII) with placental microsomal fractions in the presence of $NAD^+$ for 45 min led to a quantitative conversion of the substrate into androst-4-ene-3,17-dione (XIV; Scheme 5; R = O). This conversion involves not only the required modifications in rings A and B, but also the oxidation of the 17$\beta$-hydroxy group to the corresponding ketone by 17$\beta$-hydroxy steroid dehydrogenase known to be present in the microsomal fraction. In the light of this information the subsequent incubations with stereospecifically labelled substrates were carried out for shorter periods in the presence of a trap of 17$\beta$-hydroxyandrost-4-en-3-one. This allowed, from a single experiment, the isolation of the unconverted substrate, its C-17 oxidation analogue, 3$\beta$-hydroxyandrost-4-en-17-one and the two potential products 17$\beta$-hydroxyandrost-4-en-3-one (XIV; R = -H, -OH) and androst-4-ene-3,17-dione (XIV; R = O). Table 1 shows that when [4-$^{14}$C,4$\beta$-$^3$H]androst-5-ene-3$\beta$,17$\beta$-diol, was incubated for 30 min and processed by the methods detailed in the Experimental section, the recovered androst-5-ene-3$\beta$,17$\beta$-diol and its C-17 oxidative product, 3$\beta$-hydroxyandrost-5-en-17-one (VII; R = O) had within 3% the same $^3H/^{14}C$ ratio as the parent substrate, whereas the losses of $^3$H from the oxidation–isomerization products, androst-4-ene-3,17-dione (XIV; R = O) and 17$\beta$-hydroxyandrost-4-en-3-one (XIV; R = -H, -OH) were 92 and 90% respectively. The considerable loss of $^3$H from C-4 above indicates that the placental-isomerase-catalysed transformation involves a stereospecific labilization of the hydrogen atom in the 4$\beta$-position and contrasts with equivocal results reported in previous studies with rat liver, rat adrenal-gland or human isomerases (this aspect is critically discussed by Talalay & Benson, 1972).

The conclusion above was confirmed (Table 1) by using the 4$\alpha$-$^3$H-labelled substrate (VIIb), when the $^3$H was completely retained in the product (XIV; R = O). To prove that the $^3$H in the product (XIV; R = O) was undisturbed and still resident at C-4, the biosynthetic androst-4-ene-3,17-dione ($^3H/^{14}C = 6.24$) was hydrogenated to obtain the isomeric dihydroketones with 94% retention of $^3$H. The $^3$H was, however, removed to the extent of 90% when the ketones were subjected to an equilibration




Scheme 5. *Stereochemical status of hydrogen atoms at C-4 and C-6 in the isomerase-catalysed conversion of compound (XIII) into compound (XIV)*

Stages (VII) → (XIII) and (XIII) → (XIV) were catalysed respectively by the 3β-hydroxy steroid dehydrogenase and the 3-oxo steroid $\Delta^4$–$\Delta^5$-isomerase present in the placental microsomal fractions. The $\Delta^4$-3-oxo steroid (XIV) was then hydroxylated in the 6β-position by the fungus *Rhizopus stolonifer* to give compound (XV). Oxidation of compound (XV) with Jones reagent produced the corresponding 6-oxo steroid (XVI).

under alkaline conditions, thus showing that the $^3H$ must be located at one of the two α-positions (C-2 or C-4) with respect to the C-3 carbonyl group. The assertion then that the $^3H$ in the biosynthetic androst-4-ene-3,17-dione was at C-4 rather than C-2 rests on chemical intuition rather than firm experimental evidence.

### *Complications arising from non-enzymic isomerization of the $\Delta^5$-3-oxo steroids during the biological transformation*

It is our view that difficulties encountered by workers in previous studies on the elucidation of the mechanism of the mammalian isomerases stems from non-enzymic contributions to the isomerization process [see Talalay & Benson (1972)]. In our experiments with the placental enzyme, such a contribution must be minimal, since the previous work of Malhotra & Ringold (1965) has shown that non-enzymic isomerization favours the loss of the 4α hydrogen atoms from the $\Delta^5$-3-oxo steroids. Thus, had there been any significant non-enzymic isomerization during the incubation period, the oxidation-isomerization products of [4-$^{14}$C,4α-$^3$H]-androst-5-ene-3β,17β-diol would have shown a decrease in the $^3H/^{14}C$ ratio relative to that of the parent substrate; this was not observed.

Indeed, it is our experience that older preparations of the placental microsomal fraction, which gave poor conversions of androst-5-ene-3β,17β-diol into androst-4-ene-3,17-dione or 17β-hydroxyandrost-4-en-3-one also showed higher retention of $^3H$ (up to 30%) in the product when [4β-$^3$H]-androst-5-ene-3β,17β-diol was used as the substrate. It seems that $\Delta^5$-3β-hydroxy steroid dehydrogenase is less sensitive to denaturation than is the isomerase, and, when the activity of the latter enzyme is lower than that of the former, a substantial proportion of the $\Delta^5$-3-ketone (XIII) formed in the first step (Scheme 5) is converted into the $\Delta^4$-3-ketone (XIV) non-enzymically. Such a process will be expected to be attended by the loss of the 4α hydrogen atom, consequently enriching the product in $^3H$. Thus a prerequisite for the type of approach used in the present study is that the rate of the enzyme-catalysed isomerization reaction is equal to, or faster than, that of the dehydrogenase reaction, so that the accumulation of the $\Delta^5$-3-ketone (XIII) during the incubation, and hence its conversion into the $\Delta^4$-3-ketone (XIV) by a non-enzymic reaction, is avoided.

### *Stereochemistry of hydrogen addition to C-6 during the isomerase-catalysed reaction*

Next, the stereochemistry of the insertion of the incoming hydrogen atom at C-6 was studied. For this purpose [6-$^3$H]androst-5-ene-3β,17β-diol was synthesized chemically and after admixture with [$^{14}$C]androst-5-ene-3β,17β-diol the doubly labelled sample was converted into [4-$^{14}$C,6-$^3$H]androst-4-

Table 2. *Stereochemistry of hydrogen addition to C-6 in the isomerase-catalysed reaction*

[4-$^{14}$C,6-$^3$H]Androst-5-ene-3$\beta$,17$\beta$-diol was converted into [4-$^{14}$C,6-$^3$H]androst-4-ene-3,17-dione by microsomal oxidation/isomerization as described in the Experimental section. The product was then incubated for 24 h with a 48 h growth of *R. stolonifer*, and the 6$\beta$- and 11$\alpha$-hydroxyandrost-4-ene-3,17-dione were isolated and their $^3$H/$^{14}$C ratios measured. The 6$\beta$- and 11$\alpha$-hydroxy products were chemically oxidized to their corresponding ketones. The Roman numerals refer to structures in the Schemes.

| Microsomal incubation | $^3$H/$^{14}$C ratio |
|---|---|
| Androst-5-ene-3$\beta$,17$\beta$-diol (VII; R = -H, -OH) | 19.10 |
| Androst-4-ene-3,17-dione (XIV; R = O) | 18.61 |
| Fungal hydroxylation | |
| Androst-4-ene-3,17-dione (XIV, R = O) (re-isolated) | 17.51 |
| 6$\beta$-Hydroxyandrost-4-ene-3,17-dione (XV; R = O) | 16.49 |
| 11$\alpha$-Hydroxyandrost-4-ene-3,17-dione (XV; R = O) | 20.28 |
| Chemical oxidation of biosynthetic hydroxy compounds | |
| 6-Oxoandrost-4-ene-3,17-dione (XVI; R = O) | 0.50 |
| 11-Oxoandrost-4-ene-3,17-dione | 17.44 |

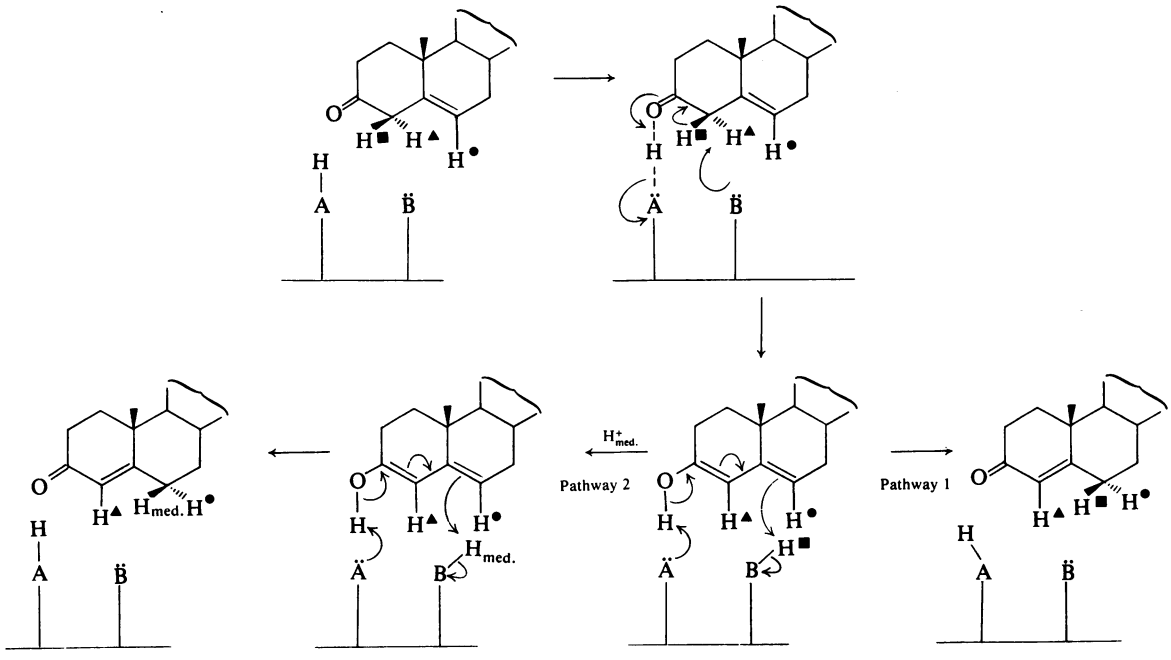
ene-3,17-dione by the placental microsomal fraction. Table 2 shows that virtually all the $^3$H present in the precursor was retained during its conversion into androst-4-ene-3,17-dione, the loss not exceeding 2.5%.

To determine the stereochemistry of $^3$H at C-6, the biosynthetic androst-4-ene-3,17-dione was incubated with broth cultures of *R. stolonifer*, a fungus known to hydroxylate androst-4-ene-3,17-dione either in the 6- or 11-positions (Eppstein *et al.*, 1954). At the end of the incubation, the steroids were extracted and purified by t.l.c. The band corresponding to the major product of hydroxylation, which had been previously characterized as 11$\alpha$-hydroxyandrost-4-ene-3,17-dione had approximately the same $^3$H/$^{14}$C ratio as had the androst-4-ene-3,17-dione originally incubated. The bands in the positions of the authentic samples of 6$\beta$-hydroxyandrost-4-ene-3,17-dione and unmetabolized androst-4-ene-3,17-dione were eluted and the material co-crystallized after the addition of carrier. The determination of radioactivity showed that these compounds (XIV and XV; R = O) retained 89 and 94% respectively of the original $^3$H. The labilization of about 6% of the $^3$H from the unconverted androst-4-ene-3,17-dione suggests that a part of the loss in the conversion androst-4-ene-3,17-dione → 6$\beta$-hydroxyandrost-4-ene-3,17-dione may be due to exchange of the labile C-6 hydrogen atoms of these compounds with the protons of the medium. Confirmation that the $^3$H and the hydroxy groups in the 6$\beta$-hydroxyandrost-4-ene-3,17-dione were in fact located at the same C atom was provided by its oxidation with Jones reagent to the corresponding 6-ketone (XVI; R = O), when the expected removal of $^3$H occurred to the extent of 99%. That the loss of $^3$H was not due to an exchange with the medium under the acidic conditions of oxidation was shown when the biosynthetic 11$\alpha$-hydroxy[6-$^3$H]androst-4-ene-3,17-dione was converted into the 11-ketone with the retention of at least 86% of the $^3$H.

On the justified assumption that the biological hydroxylation reaction occurs with the retention of configuration, the preservation of most of the C-6 $^3$H during the 6$\beta$-hydroxylation suggests that the precursor androst-4-ene-3,17-dione contained the $^3$H in the 6$\alpha$-orientation. It therefore follows that, in the isomerase-catalysed migration of the $C_{(5)}$=$C_{(6)}$ double bond, the incoming hydrogen atom from an acidic group on the enzyme must enter C-6 from the $\beta$-face, forcing the $^3$H into the $\alpha$-position.

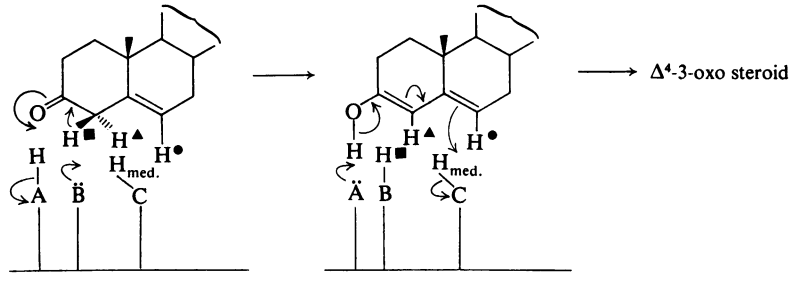
### *Mechanistic considerations*

In their classical work, Talalay and co-workers (Talalay *et al.*, 1952; Talalay & Wang, 1955; Talalay & Benson, 1972) showed that the reaction catalysed by the bacterial 3-oxo steroid $\Delta^4$–$\Delta^5$-isomerase involves an intramolecular migration of the 4$\beta$-hydrogen atom to the 6$\beta$-position and proposed a mechanism in which the 4$\beta$-hydrogen atom from the steroid nucleus was removed by a histidine residue of the enzyme and then introduced at C-6, presumably after the rotation of the imidazolium ring. These conclusions were confirmed and extended by Malhotra & Ringold (1965), who, on the basis of kinetic evidence and by analogy with the mechanism operating in non-enzymic isomerizations, emphasized that the reaction occurs via an enzyme–enol and not an enzyme–enolate intermediate. The assertion necessitates that the enzyme, in addition to a basic group for the abstraction of the C-4$\beta$-hydrogen atom, should also contain an acidic group in the vicinity of the substrate oxygen for the generation of an enol. An abbreviated form of the mechanism postulated by Malhotra & Ringold (1965) incorporating these features is shown in Scheme 6 (Pathway 1). A similar intramolecular hydrogen migration has been observed with the 3-oxo steroid $\Delta^4$–$\Delta^5$-isomerase activity associated with the cholesterol oxidase complex of *Nocardia erythropolis* (Smith & Brooks, 1977).




Scheme 6. *Mechanistic alternatives for an enzymic-isomerization process using a single catalytic group for proton-removal and -addition steps*

Pathway 1 gives rise to an intramolecular hydrogen transfer as is observed with the *P. testosteroni* enzyme. In pathway 2 the group –B–H■ protonates C-6 after undergoing exchange with the proton of the medium ($H^+_{med.}$).




Scheme 7. *Hypothetical mechanism for the isomerization reaction involving two different groups for proton-removal and -addition steps*

Cumulatively, the results described in the present paper show that, unlike the microbial isomerases, the 3-oxo steroid $\Delta^4$–$\Delta^5$-isomerase of human placenta does not catalyse the double-bond migration through an intramolecular hydrogen-transfer mechanism. With both types of enzymes, however, the stereochemistry of the hydrogen elimination from C-4 of the substrate and the new C–H bond formation at C-6 of the product are identical.

The demonstration of an intramolecular hydrogen transfer with the *P. testosteroni* enzyme led to the attractive as well as reasonable suggestion that the same enzymic group may be involved in both the C-4-deprotonation and C6-reprotonation steps. For the placental enzyme, in which an elimination–addition profile is observed, two mechanistic possibilities exist. Either the reaction may be rationalized in terms of a mechanism involving two groups [a basic group participating in proton removal from C-4 and an acidic group in proton addition to C-6 (Scheme 7)], or alternatively one could argue that a mechanism similar to that for the bacterial enzyme also operates for the placental enzyme, except that in the reaction catalysed by the latter enzyme the rate of exchange of the hydrogen in –B–H with the protons of the medium (Scheme 6, Pathway 2) is faster than the rate of rearrangement of the enzyme–enol complex to give the conjugated ketone.

Thus, with respect to intramolecular as against exchange-mediated hydrogen transfer, the duality of mechanistic behaviour shown by 3-oxo steroid $\Delta^4$–$\Delta^5$-isomerases parallels that for the aldose–ketose isomerases [for reviews on the latter group of enzymes, see Akhtar & Jones (1978) and Rose (1975)].

M. A. thanks the Medical Research Council for the generous support of this work.

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