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Chemical and Enzymic Studies on the Characterization of Intermediates during the Removal of the 14α-Methyl Group in Cholesterol Biosynthesis

THE USE OF 32-FUNCTIONALIZED LANOSTANE DERIVATIVES

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By using cell-free preparations of rat liver it was shown that the removal of the 14α-methyl group (C-32) of steroids containing either a $\Delta^{7(8)}$ or a $\Delta^{8(9)}$ double bond is attended exclusively by the formation of the corresponding 7,14- and 8,14-dienes respectively (structures of types III and VIII). Cumulative evidence from a variety of experimental approaches has led to the deduction that $\Delta^{8(14)}$-steroids are not involved as intermediates on the major pathway of cholesterol biosynthesis. The metabolism of [32-^{3}H]lanost-7-ene-3β,32-diol (structure of type I) results in the formation of radioactive formic acid, no labelled formaldehyde being formed. By using appropriately labelled species of the compound (I) it was found that the release of formic acid and the formation of 4,4-dimethylcholesta-7,14-dien-3β-ol (structure of type III) were closely linked processes, and that in the conversion of compound (I) into compound (III), 3β-hydroxylanost-7-en-32-al (II) is an obligatory intermediate. Both the conversion of lanost-7-ene-3β,32-diol (I) into 3β-hydroxylanost-7-en-32-al (II) and the further metabolism of the latter (II) to 4,4-dimethylcholesta-7,14-dien-3β-ol (III) exhibited a requirement for NADPH and O_2. This suggests that the oxidation of the 32-hydroxy group of compound (I) to the aldehyde group of compound (II) does not occur by the conventional alcohol dehydrogenase type of reaction, but may proceed by a novel mechanism involving the intermediacy of a *gem*-diol. A detailed overall pathway for the 14α-demethylation in cholesterol biosynthesis is considered, and proposals about the mechanism of individual steps in the pathway are made.

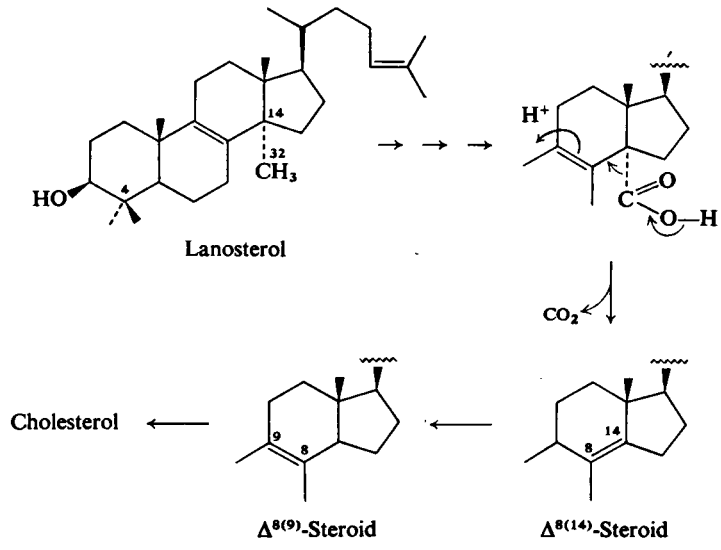
The mechanism by which the 14α-methyl group (C-32) of lanosterol is removed in the biosynthesis of cholesterol has been the subject of much discussion in the last two decades (for general reviews in the steroid field, see Rees & Goodwin, 1972, 1973, 1974; Mulheirn, 1975). The first formal view on this subject assumed that the transformation involves an initial oxidation of the 14α-methyl group to the corresponding carboxylic acid (Scheme 1), which, utilizing the activation provided by the presence of a βγ double bond at position 8,9, undergoes decarboxylation to produce a $\Delta^{8(14)}$-steroid (Richards & Hendrickson, 1964). The latter intermediate after isomerization to a $\Delta^{8(9)}$-desmethyl sterol enters the biosynthetic pathway for further nuclear and side-chain modifications (for general reviews in the steroid field, see Rees & Goodwin, 1972, 1973, 1974; Mulheirn, 1975).

Subsequent experiments (Canonica *et al.*, 1968*a*,*b*; Gibbons *et al.*, 1968; Akhtar *et al.*, 1968, 1969*a*,*b*; Goodwin, 1971, and references cited therein), which showed that the removal of the 14α-methyl group is attended by the labilization of the 15α-hydrogen atom of lanosterol, posed new problems and gave rise to several interesting speculations about the mechanism for the C-14–C-32 bond-cleavage step (Goodwin, 1971; Watkinson *et al.*, 1971; Akhtar *et al.*, 1972; Fiecchi *et al.*, 1972; Schroepfer *et al.*, 1972; Spike *et al.*, 1974, and references cited therein). In the present paper we critically examine the current status of knowledge in the area and describe experiments on the nature of chemical events that occur at C-32 and C-15 before the loss of the carbon atom.

Experimental

Materials

Chemicals were generally obtained from BDH, Poole, Dorset, U.K., except for the following special chemicals, which were obtained from the sources indicated: Tween 80 and cholesterol (Koch–Light, Colnbrook, Bucks., U.K.); NADP and glucose 6-



Scheme 1. *Classical pathway for the removal of C-32*

phosphate dehydrogenase [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.]; silica gel for t.l.c. (E. Merck A.-G., Darmstadt, Germany); butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (CIBA, Basel, Switzerland); NaB^3H_4 and ^3H_2O (The Radiochemical Centre, Amersham, Bucks., U.K.).

Measurement of radioactivity

The radioactivity of samples was counted in butyl-PBD in toluene (10ml, 8g/litre) at 12°C in an Intertechnique SP 60 liquid-scintillation counter, programmed to automatic quench correction. Values recorded in c.p.m. were counted at an efficiency of 57.8%. Values recorded in d.p.m. have been corrected to 100% efficiency.

Thin-layer chromatography

For the preparation of analytical t.l.c. plates, a slurry of silica gel GF_{254} (50g/100ml) in water or $AgNO_3$ solution was evenly spread over the glass plates (20cm×10cm) to a thickness of 0.4mm. After drying in air the plates were activated at 100°C for 1h and then stored over $CaCl_2$ at room temperature (18°C). Preparative t.l.c. plates were prepared as above, but silica gel PF_{254} to a thickness of 2mm was used.

Rat liver preparations and incubation procedures

(1) *Crude rat liver homogenate.* Male Wistar albino rats (100–150g) were killed by cervical dislocation, and the excised livers were homogenized in cold (1–4°C) 0.1M-potassium phosphate buffer, pH7.3, containing 30mM-nicotinamide and 4mM-$MgCl_2$ (1.25ml/g of liver). The homogenization consisted of 60 strokes in a loose-fitting piston-barrel-type homogenizer. Cell debris and mitochondria were sedimented by centrifugation at 10000*g* for 15min at 1–4°C, after which the supernatant was filtered through glass wool to give the crude rat liver homogenate used in the incubations.

(2) *Subcellular fractions.* The crude homogenate was centrifuged for 60min at 105000*g*. The 105000*g* supernatant was then decanted off and the remaining microsomal pellet was rinsed, resuspended in the same volume of buffer from which it had sedimented and re-centrifuged for 60min at 105000*g*. The supernatant was then decanted off and the microsomal pellet was rinsed with cold buffer and resuspended in the same volume of buffer from which it had sedimented. This preparation was used in the washed microsomal incubations.

All operations in the above preparations were performed at 1–4°C.

(3) *Incubation procedures.* Sterols were dissolved in acetone (1ml) containing Tween 80 (25mg). The acetone was then removed with a stream of N_2 and the residue emulsified in water (0.5ml) before addition of the rat liver preparation. The crude rat liver homogenates were supplemented with glucose 6-phosphate (1.0mM) and reduced glutathione (1.0mM). For the microsomal preparations, any NADPH generator added contained glucose 6-phosphate (4μmol) and glucose 6-phosphate dehydrogenase

(0.6unit) per μmol of $NADP^+$. Incubations were performed in 50ml Erlenmeyer flasks at 37°C with gentle shaking. Aerobic conditions were obtained by incubating in unstoppered flasks, and anaerobic conditions were obtained by passing purified N_2 through the incubation flasks. The N_2 was purified from any O_2 contamination by bubbling through a solution of sodium anthraquinone-β-sulphonate (2g)+sodium dithionite (15g)+KOH (20g) in water (100ml) and was then dried over $CaCl_2$.

Further details of the incubation conditions are described below.

Isolation and purification of cholesterol as the 5,6-dibromide derivative

Incubations were terminated by the addition of methanolic 10% (w/v) KOH (5ml). Non-radioactive carrier cholesterol (100mg) was then added and the mixture was saponified by heating for 5min on a steam bath. The cholesterol was extracted with ether (3×25ml), and the combined ether extracts were washed with water (3×25ml) and saturated NaCl solution (25ml). After drying over anhydrous Na_2SO_4 the solvent was removed under reduced pressure. The solid residue was dissolved in ether (1.5ml) and to this was added 1ml of Fieser's (1953) solution [bromine (2ml)+acetic acid (30ml)+sodium acetate (200mg)]. The mixture was cooled in ice for 15min and the crystallized cholesterol dibromide was washed with cold methanol. Crystallization (three times) from ether/methanol gave pure cholesterol dibromide (70mg, m.p. 111–113°C).

Extraction and isolation of other sterol metabolites

Incubations were terminated by the addition of methanolic 10% (w/v) KOH (5ml). The appropriate non-radioactive carrier sterols (2mg) were then added and the mixture was saponified by heating for 5min on a steam bath. The sterols were extracted with ether (3×25ml) and the combined ether extracts were washed with water (3×25ml) and saturated NaCl solution (25ml). After drying over anhydrous Na_2SO_4, the solvent was removed under reduced pressure. The appropriate sterols were then isolated and purified by t.l.c.

In the experiments of Tables 3, 6 and 7, 4,4-dimethylcholest-7,14-diene-3β-ol was isolated first by t.l.c. on preparative silica-gel PF_{254} impregnated with 10% $AgNO_3$ with chloroform/diethyl ether/acetic acid (194:5:1, by vol., solvent system I) and then the band of R_F 0.28 was eluted with diethyl ether and the material converted into the corresponding acetate by treatment with acetic anhydride (0.2ml) and pyridine (2.0ml) at 20°C for 18h. The acetate was further subjected to t.l.c. on silica gel (PF_{254}) impregnated with 10% (w/v) $AgNO_3$ with chloroform/light petroleum (b.p.60–80°C)/acetic acid (50:100:1, by vol.), and the band of R_F 0.5 was eluted and used for co-crystallization. To assess the incorporation of radioactivity from precursors into 4,4-dimethylcholest-8(14)-en-3β-ol, the sterols were first separated with solvent system I (see above), and then the band of R_F value 0.48 was acetylated, treated with 3-chloroperbenzoic acid and processed as described under 'Characteristic epoxidation product of 4,4-dimethyl-8(14)-en-3β-ol acetate'. In the experiments of Table 3 and 7, 3β-hydroxylanost-7-en-32-al was separated by t.l.c. on silica gel (PF_{254}) in chloroform/ether/acetic acid (194:5:1, by vol.). The band of R_F 0.60 was rechromatographed as above in light petroleum/acetone (8:2, v/v) to give the purified compound, with R_F 0.46.

Analysis of incubations for radioactive formic acid, water and formaldehyde

(1) *Acid-volatile distillate.* Incubations were terminated by the addition of redistilled non-radioactive formic acid (60mg), formaldehyde (40mg) and orthophosphoric acid (0.5ml, relative density 1.75). The mixture was then transferred to a round-bottomed 100ml flask and freeze-dried under high vacuum, the acid-volatile distillate being collected in a liquid-N_2 trap. Water (1.0ml) was added to the residue, which was again freeze-dried. A portion (0.2ml) of the total acid-volatile distillate was counted for radioactivity in methanol/0.8% butyl-BPD in toluene (2:5, v/v) to determine the amount of [^{3}H]formic acid+^3H_2O (+[^{3}H]formaldehyde if any) present.

(2) *Radioactivity in formic acid and water.* One-half of the acid-volatile distillate was titrated with 1M-NaOH, with phenolphthalein as indicator, to form the non-volatile sodium salt of formic acid and to check the recovery of formic acid from the previous freeze-drying procedure (96–100%). The volatile water and formaldehyde were removed with a rotary vacuum pump and collected in a liquid-N_2 trap. Water (2ml) was then added to the residual sodium formate and evaporated as above. A portion (0.2ml) of the total distillate was counted for radioactivity in methanol/0.8% butyl-PBD in toluene (2:5, v/v, 14ml) to determine the amount of ^3H_2O (+[^{3}H]formaldehyde if any, see below) present. Methanol (2×5ml) was added to the residual sodium formate and removed under vacuum. The sodium formate was then dissolved in methanol/water (99:1, v/v; 20.0ml) and a portion (3.0ml) was counted for radioactivity in butyl-PBD in toluene (10ml).

In some cases (see the text) the sodium formate was recrystallized from methanol/ether and then converted into *p*-bromophenacyl formate (see below).

(3) *Radioactivity in formaldehyde.* To one-half of the acid-volatile distillate was added 40ml of

dimedone reagent [recrystallized dimedone (5,5-dimethyl-1,3-cyclohexanedione; 500mg)+Na_2HPO_4 (465mg) + $NaH_2PO_4,2H_2O$ (350mg) in water (100ml)]. The formaldehyde–dimedone complex precipitated in the cold and was filtered off, washed with water and crystallized (twice) from methanol/water. After drying at 50°C under vacuum, a sample (40–45mg) was accurately weighed and counted for radioactivity in butyl-PBD in toluene (10ml).

Chemical conversion of sodium formate into p-bromophenacyl formate

Sodium formate (204mg) was dissolved in water (3ml) and treated with *p*-bromophenacyl bromide (831mg) in ethanol (9ml) and the mixture was refluxed for 30min. The ethanol was then removed under reduced pressure and the product extracted with ether. The ether extract was washed with water and saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was applied to a silica-gel-PF_{254}-coated t.l.c. plate and developed in benzene/ethyl acetate (19:1, v/v). The band corresponding to *p*-bromophenacyl formate, R_F 0.43, was eluted and crystallized (twice) from dry ethanol (yield 87mg).

Characteristic epoxidation product of 4,4-dimethylcholest-8(14)-en-3β-ol acetate

Either 4,4-dimethyl[3α-^{3}H]cholest-8(14)en-3β-ol acetate (5mg; 270000 c.p.m.) and dihydrolanosterol acetate (5mg) or [3α-^{3}H]dihydrolanosterol acetate (5mg; 220000 c.p.m.) and 4,4-dimethylcholest-8(14)-en-3β-ol acetate (5mg) were dissolved in anhydrous ether (4ml) and treated with 3-chloroperbenzoic acid (15mg) in anhydrous ether (2ml) at 0°C. In each case the reaction was continued for 60min at 0°C and the mixture was then diluted with ether and washed with cold 0.5M-NaOH, water and saturated NaCl solution. After drying over anhydrous Na_2SO_4 the solvent was removed under reduced pressure. Analytical t.l.c. in chloroform/light petroleum/acetic acid (50:100:1, by vol.) of the residues revealed that 42% of the 4,4-dimethyl[3α-^{3}H]cholest-8(14)-en-3β-ol acetate had been converted into a characteristic epoxidation product of R_F 0.22.

Preparation of 4,4-dimethylcholesta-8,14-dien-3β-ol and 4,4-dimethylcholesta-7,14-dien-3β-ol

These two compounds and their corresponding acetates were prepared as described previously (Akhtar *et al.*, 1969*b*; Watkinson *et al.*, 1971) by methods adapted from those of Gautschi & Bloch (1958) and Knight *et al.* (1966).

Chemical conversion of 4,4-dimethylcholesta-7,14-dien-3β-ol acetate (structure of type III) into 4,4-dimethylcholest-8(14)-ene-3β,7ξ,15ξ-triol (IV) by the method of Woodward et al. (1957)

4,4-Dimethylcholesta-7,14-dien-3β-ol acetate (structure of type III) (55mg) was dissolved in anhydrous ether (2.3ml) and treated with a solution of perphthalic acid (25.5mg) in anhydrous ether (0.8ml). The reaction mixture was left at room temperature (20°C) for 45h, after which it was extracted with ether, washed with 0.1M-NaOH, water and saturated NaCl solution and dried over anhydrous Na_2SO_4. The ether was then removed under reduced pressure and the residue dissolved in a solution of KOH (215mg) in 95% (v/v) ethanol (7.5ml) and refluxed for 3h. The ethanol was subsequently removed under reduced pressure and the product washed with water, a little ice-cold methanol and dried. Crystallization (three times) from chloroform gave pure 4,4-dimethylcholest-8(14)-ene-3β,7ξ,15ξ-triol (IV), yield 20.8mg, m.p. 240°C.

Preparation of 4,4-dimethylcholest-8(14)-en-3β-ol (VII) by the method of Gautschi & Bloch (1958)

A solution of 4,4-dimethylcholesta-5,7-dien-3β-ol (1g) in ethyl acetate (50ml) was stirred under an atmosphere of H_2 in the presence of 10% (w/w) palladium on charcoal (500mg) at room temperature. The hydrogenation was continued for 4h, after which the catalyst was removed by filtration through kieselguhr. Removal of the solvent under reduced pressure gave a solid product that showed no u.v. absorption. Crystallization (twice) from ether/methanol gave pure 4,4-dimethylcholest-8(14)-en-3β-ol (VII), yield 686mg, m.p. = 141°C, $[\alpha]_D$ = +14.6°.

4,4-Dimethyl[3α-^{3}H]cholest-8(14)-en-3β-ol (VIIa)

4,4-Dimethylcholest-8(14)-en-3β-ol (VII) (100mg) in acetone (20ml) was stirred for 5min under N_2 and then Jones reagent (Bowden *et al.*, 1946) (0.1ml) was added dropwise. After 2min a few drops of methanol were added to decompose any excess Jones reagent. Filtration through cottonwool was then followed by addition of water and the ether extraction and drying of the product, which showed the characteristic keto-group absorption in the i.r.-absorption spectrum. Crystallization once from ether/methanol gave 4,4-dimethylcholest-8(14)-en-3-one, yield 65mg.

NaB^3H_4 (1mg, 2mCi) was added to a solution of 4,4-dimethylcholest-8(14)en-3-one (50mg) in methanol (20ml). After 3h, excess $NaBH_4$ was added and the reaction mixture was left for a further 3h. Water was then added and the precipitated product was extracted with ether and dried in the usual manner. Purification by preparative t.l.c. in chloroform (developed twice) gave pure 4,4-dimethyl-

[3α-^{3}H]cholest-8(14)-en-3β-ol (VIIa), R_F 0.53, yield 29mg; sp. radioactivity 9.12×10^6 c.p.m./mg.

4,4-Dimethyl[2-^{3}H]cholest-8(14)-en-3β-ol (VIIb)

4,4-Dimethylcholest-8(14)-en-3-one (50mg) was equilibrated overnight in ethanolic 2% (w/v) KOH (10ml) in the presence of ^3H_2O (0.1ml, 60mCi). Excess water was added and the precipitated 4,4-dimethyl[2-^{3}H]cholest-8(14)-en-3-one was extracted with ether and dried in the usual manner. It was then reduced with excess $LiAlH_4$ and the 4,4-dimethyl[2-^{3}H]cholest-8(14)-en-3β-ol (VIIb) was isolated by preparative t.l.c. in chloroform (developed twice), R_F 0.53, yield 10mg; sp. radioactivity 7.43×10^5 c.p.m./mg. The acetylation of 4,4-dimethylcholest-8(14)-en-3β-ol as well as its variously labelled counterparts was performed by treating a solution of the corresponding alcohol (2–5mg) in pyridine (2.0ml) with acetic anhydride (0.2ml). The reaction mixture was left at 20°C for 18h and then diluted with water. The product was extracted with diethyl ether (2×30ml). The organic phase was washed with water (3×20ml), dried (with anhydrous Na_2SO_4) and evaporated to dryness under reduced pressure. The product was purified, if necessary, by t.l.c. as described under 'Extraction and isolation of other sterol metabolites'.

Preparation of labelled dihydrolanosterol

[3α-^{3}H]- or [2-$^3H_2$]-dihydrolanosterol was prepared in the same manner as compounds (VIIa) and (VIIb) respectively from unlabelled dihydrolanosterol. The latter compound was prepared from lanosterol (now commercially available from Koch–Light, Colnbrook, Bucks., U.K.) by acetylation to give lanosterol acetate (see above for the method of acetylation) followed by hydrogenation as described below.

Platinum oxide (40mg) was reduced in acetic acid (10ml) with hydrogen, and to this was added a solution of lanosterol acetate (200mg) in acetic acid (30ml). The mixture was hydrogenated at room temperature and atmospheric pressure (101325Pa) for 2.5h, during which time 1mol of hydrogen was smoothly taken up. The resulting mixture was filtered, and the filtrate evaporated to dryness. The solid residue was crystallized three times from methanol to give pure dihydrolanosterol acetate (150mg). This was reduced with excess $LiAlH_4$ to yield, after two crystallizations from diethyl ether/methanol, dihydrolanosterol, 100mg, m.p. 138°C.

Preparation of 3β-hydroxy[3α-^{3}H]lanost-7-en-32-al (IIb) and [3α-^{3}H]- or [32-^{3}H]-lanost-7-ene-3β,32-diol (Ib or Ia)

3β-Hydroxylanost-7-ene nitrile. 3β-Acetoxylanost-7-ene 32-nitrile (100mg), synthesized via nitrite photolysis (Batten *et al.*, 1972), was dissolved in methanol (16ml) and treated with 10% (w/v) methanolic KOH (1.6ml). After 10h, water was added and the product was extracted with ether, washed and dried. Removal of the solvent under reduced pressure gave 3β-hydroxylanost-7-ene 32-nitrile (yield 90mg; R_F 0.31 on silica-gel PF_{254} t.l.c. plates developed in chloroform).

3β-Hydroxy[3α-^{3}H]lanost-7-ene 32-nitrile. ^{3}H was introduced into the 3α-position by the procedure previously described for 4,4-dimethylcholest-8(14)-en-3β-ol, involving oxidation with Jones reagent of 3β-hydroxylanost-7-ene 32-nitrile (60mg) followed by the reduction of the resultant 3-ketone with NaB^3H_4. Preparative t.l.c. in chloroform gave the pure 3β-hydroxy[3α-^{3}H]lanost-7-ene 32-nitrile (R_F 0.31, yield 50mg).

3β-Hydroxy[3α-^{3}H]lanost-7-en-32-al. 3β-Hydroxy [3α-^{3}H]lanost-7-ene 32-nitrile (50mg) was dissolved in dry tetrahydrofuran (3.4ml). The solution was stirred, and small portions of $LiAlH_4$ (70mg) were carefully added at such a rate that with cooling the temperature did not rise above about 20°C. The reaction mixture, sealed from the moisture in the air, was stirred at room temperature for 70h. It was then cooled on an ice bath and the excess of $LiAlH_4$ decomposed with ethyl acetate. Next, 0.2M-HCl (4ml) was added and after 15min at room temperature the products were extracted with ether, washed with 10% (w/v) $NaHCO_3$ solution, water and saturated NaCl solution. After drying over anhydrous Na_2SO_4 the solvent was evaporated under reduced pressure. The residual oil was applied to silica-gel t.l.c. plates and developed seven times in light petroleum/acetone (9:1, v/v) (R_F of 3β-hydroxylanost-7-ene 32-nitrile = 0.30, R_F of 3β-hydroxylanost-7-en-32-al = 0.40) to give pure 3β-hydroxy-[3α-^{3}H]lanost-7-en-32-al (IIb), yield 21.4mg, m.p. 128°C, sp. radioactivity 39.6×10^6 d.p.m./mg.

[3α-^{3}H]Lanost-7-ene-3β,32-diol (Ib). 3β-Hydroxy-[3α-^{3}H]lanost-7-en-32-al (25mg) in methanol (10ml) was treated with excess $NaBH_4$. After standing at room temperature for 3h, water was added and the product extracted with ether. The ether phase was washed with water and saturated NaCl solution, dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure. Preparative t.l.c. in chloroform/ether/acetic acid (194:5:1, by vol.) and elution of the band of R_F 0.46 have [3α-^{3}H]lanost-7-ene-3β,32-diol (Ib), yield 20mg, m.p. 201°C, sp. radioactivity = 39.6×10^6 d.p.m./mg.

[32-^{3}H]Lanost-7-ene-3β,32-diol (configuration A). 3β-Hydroxylanost-7-en-32-al (II) (20mg) in methanol (8ml) was treated with NaB^3H_4 (3mg, 6mCi) and left at room temperature for 2h. Excess $NaBH_4$ was then added, and after 3h the product was isolated as above to give, after preparative t.l.c. in chloroform/ether/acetic acid (194:5:1, by vol),

[32-^{3}H]lanost-7-ene-3β,32-diol (Ia), R_F 0.46, yield 16mg, sp. radioactivity = 65.2×10^6 d.p.m./mg.

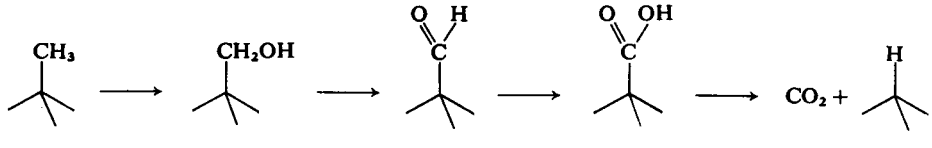
[32-^{3}H]Lanost-7-ene-3β,32-diol (configuration B). [32-^{3}H]Lanost-7-ene-3β,32-diol (configuration A) (0.4mg; 5.02×10^6 d.p.m.) in dry pyridine (0.2ml) was added to a suspension of CrO_3 (6mg) in dry pyridine (0.1ml) and left at room temperature for 4h. Water was then added, and the product was extracted with ether and washed with water and saturated NaCl solution. After drying over anhydrous Na_2SO_4 the solvent was removed under reduced pressure. Preparative t.l.c. in light petroleum/acetone (4:1, v/v) revealed that all the substrate (R_F 0.35) had been oxidized to 3-oxo[32-^{3}H]lanost-7-en-32-al (R_F 0.82). A portion of the eluted 3-oxo[32-^{3}H]lanost-7-en-32-al (0.3mg) was then reduced with excess $NaBH_4$ as above to give, after preparative t.l.c. in chloroform/ether/acetic acid (194:5:1, by vol.), [32-^{3}H]lanost-7-ene-3β,32-diol (diol B), R_F 0.46, yield 0.25mg, sp. radioactivity 6.47×10^5 d.p.m.

Results

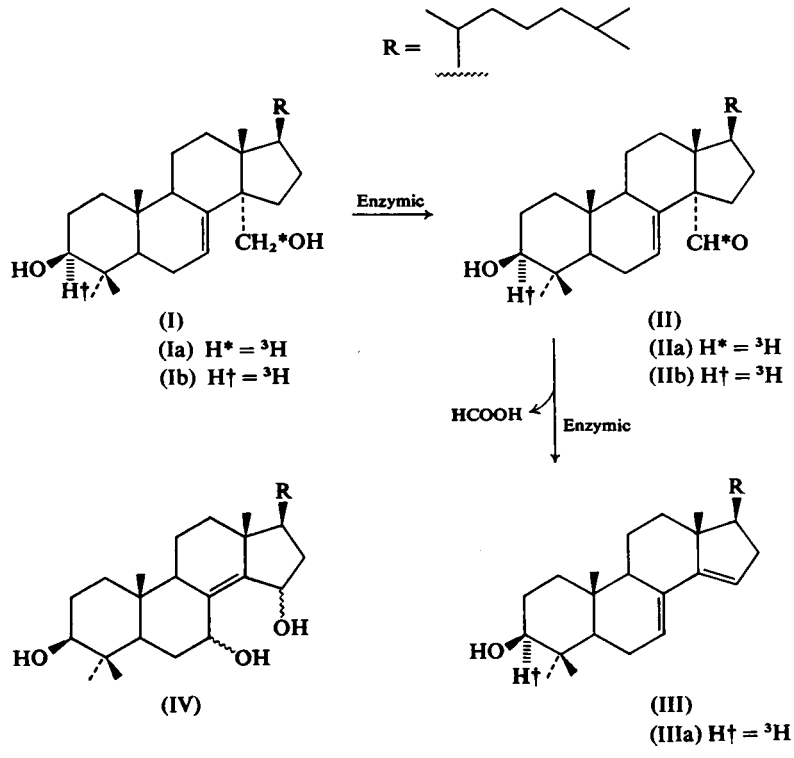
The fate of C-32

Background. Bloch and his colleagues (Olson *et al.*, 1957) showed that in the hepatic biosynthesis of cholesterol the three carbon atoms from the 4α-, 4β- and 14α-methyl groups of lanosterol were released as CO_2. In accordance with the accepted dogma at the time, it was proposed that the methyl groups are eliminated as CO_2 after their oxidation into the corresponding carboxylic acid derivatives, as shown in Scheme 2. That such a mechanism operates for C-4 demethylation was subsequently confirmed by the isolation of 4α-carboxylic acid derivatives, which were shown to be obligatory intermediates in cholesterol biosynthesis (Miller & Gaylor, 1970*a*,*b*; Hornby & Boyd, 1971; Bloxham & Akhtar, 1971). Until the work from our groups, the preliminary account of which has been published (Alexander *et al.*, 1971, 1972), the fate of the C-32 was less clearly understood. The possibility was considered that the carbon atom may be removed as formaldehyde or formate and subsequently oxidized to produce the CO_2 isolated previously (Olson *et al.*, 1957). Indeed, the presence of formaldehyde dehydrogenase in rat liver homogenate of the type normally used to study steroid biosynthesis is documented in the literature (Strittmatter & Ball, 1955), and during preliminary enquiries we have found that formate is also metabolized to CO_2 by this rat liver preparation.

Release of C-32 as formic acid during the metabolism of lanost-7-ene-3β,32-diol (I, hereafter referred to as Δ^7-3,32-diol). To monitor the fate of the 32-carbon atom during 14α-demethylation, we required 32-functionalized lanosterol derivatives that also contained radioactive label at this position. It is known that enzymes participating in the removal of the 14α-methyl group of the physiologically more important $\Delta^{8(9)}$-steroids (V; see Scheme 4 below) also use the corresponding steroids containing the double bond in the 7,8-position. Since 32-functionalized $\Delta^{7(8)}$-steroids are relatively conveniently synthesized, compounds in this series were used for the enzymic work. 3β-Hydroxylanost-7-en-32-al (II, Δ^7-aldehyde; Scheme 3) was synthesized (Batten *et al.*, 1972, and references cited therein) as described in the Experimental section and then reduced with NaB^3H_4 to give [32-^{3}H]Δ^7-3,32-diol (Ia). A sample of the latter alcohol was incubated aerobically with a washed microsomal fraction of rat liver homogenate, which was shown to be free of formaldehyde or formate oxidase activities in the presence of a NADPH-regenerating system, consisting of $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. After the addition of carrier formic acid (30.5mg) and acidification to pH2.0 with phosphoric acid, the reaction mixture was freeze-dried and the acid-volatile distillate (see the Experimental section) was shown to contain more than 80% of the steroid-bound radioactivity originally used in the incubation (Table 1). In the distillate, after neutralization with NaOH, 55% of the radioactivity was associated with H_2O and the remaining 45% co-crystallized with sodium formate. A part of the sodium formate after conversion into *p*-bromophenacyl formate retained over 85% of the radioactivity. Several types of control experiments were also performed. In one, when boiled enzyme was used, no detectable radioactivity was present in the acid-volatile distillate. In another, aerobic incubation was performed with the washed microsomal fraction, NADPH-regenerating system and the



Scheme 2. *Pathway for the oxidative elimination of a methyl group*



Scheme 3. *Metabolism of 32-oxygenated Δ^8-steroids*

Table 1. *Removal of C-32 as formate from [32-³H]lanost-7-ene-3β,32-diol (Ia)*

The [32-³H]Δ^7-3,32-diol (Ia) (50μg: 5730000 d.p.m.) was aerobically incubated for 45min with washed microsomal fraction (5ml) supplemented with NADPH-generation system consisting of 0.5mM-$NADP^+$, 2mM-glucose 6-phosphate and 3 units of glucose 6-phosphate dehydrogenase. The incubation mixture was freeze-dried and processed for the isolation of sodium formate and formaldehyde from the acid-volatile fraction as described in the Experimental section.

Addition	³H (% of the original radioactivity)		
	In the total acid-volatile distillate	In sodium formate	In formaldehyde
None	81.1	38	<0.1
Formaldehyde (150μg)	82.4	39	<0.1
None (control: boiled microsomal fraction)	5	<1.0	<1.0

[32-³H]Δ^7-3,32-diol (Ia), but in the presence of non-radioactive formaldehyde (Table 1). After 45min, 83% of the incubated radioactivity was found in the acid-volatile distillate, none of which was associated with formaldehyde isolated as its dimedone derivative. Finally, the removal of C-32 and the release of the radioactivity from the [32-³H]Δ^7-3,32-diol (Ia) in the above incubations compulsorily required NADPH and O_2; no significant reaction occurred in the presence of $NADP^+$, NAD^+ or NADPH under N_2 (Table 2, also see Table 7).

That C-32 in the above experiments was eliminated as formic acid suggested that metabolism of the Δ^7-3,32-diol (I) occurs via the intermediacy of a 32-

Table 2. *Cofactor requirements for the release of formic acid during the metabolism of the* [32-^{3}H]Δ^7-3,32-diol (Ia)
The [32-^{3}H]Δ^7-3,32-diol (Ia) (50μg; 3.26×10^6 d.p.m.) was aerobically incubated with washed microsomal fraction (5.0ml) for 45min in the presence of cofactors as indicated. In Expt. 1, NADPH was generated from 1.0mM-$NADP^+$, 4.0mM-glucose 6-phosphate and 3 units of glucose 6-phosphate dehydrogenase. The radioactive products were isolated as described in the Experimental section.

Expt. no.	Addition	^{3}H (% of original radioactivity) In acid-volatile distillate	In water	In formic acid
1	NADPH (1.0mM)	100.0	43.5	56.5
2	NAD^+ (2.0mM)	<5	—	0.4
3	None	<3	—	0.1

Table 3. *Formation of the* Δ^7*-32-aldehyde (II) from the* Δ^7*-3,32-diol (I) and the cofactor requirements for its biosynthesis*
The [3α-^{3}H]Δ^7-3,32-diol (Ib) (50μg; 1.98×10^6 d.p.m.) was incubated with washed microsomal fraction (5.0ml) for 30min in the presence of the 7,14-diene (III) (1.0mg). In each case the concentration of NADPH below corresponds to that of $NADP^+$ used in the regenerating system. Sterols were extracted with ether and isolated by t.l.c. as described in the Experimental section.

Expt. no.	Additions	Gas phase	^{3}H (% of original radioactivity) associated with Δ^7-3,32 Diol (I)	Δ^7-32-Aldehyde (II)	7,14-Diene+ 4,4-dimethyl-cholest-7-en-3β-ol (III)	Recovery from t.l.c. plate (%)
1	NADPH (0.1mM)	Air	68.5	20.2	1.2	89.9
2	NADPH (1.0mM)	Air	20	<5.0	40.0	80.0
3	$NADP^+$ (1.0mM)	N_2	84.4	1.7	0.2	86.3
4	NAD^+ (0.4mM)	N_2	88.3	1.8	0.4	90.5
5	None	Air	86.4	0.7	0.2	87.3

aldehyde (II; Scheme 3). Preliminary support for such a hypothesis was provided by the demonstration that the incubation of the [32-^{3}H]Δ^7-3,32-diol (Ia; 50μg; 2.7×10^6 d.p.m.) in the presence of increasing amounts of non-radioactive Δ^7-32-aldehyde (II; 0, 200 and 400μg respectively) led to a progressive decrease in the appearance of radioactivity into formate (45, 28 and 18% respectively of the original radioactivity in compound 1a). This may be explained if it is assumed that the [32-^{3}H]Δ^7-aldehyde (IIa) formed from the [32-^{3}H]Δ^7-3,32-diol (Ia) was diluted by the non-radioactive trap, thus resulting in a decrease in the specific radioactivity of the formate ultimately eliminated. The experiments of Table 3 provided more direct evidence for the formation of the Δ^7-32-aldehyde under the conditions of the biosynthesis. Thus 20% of the radioactivity from the [3α-^{3}H]Δ^7-3,32-diol (Ib) accumulated in the Δ^7-32-aldehyde when the incubation was performed in the presence of a limiting supply of $NADPH+O_2$. The oxidized form of nicotinamide nucleotides were unable to support the formation of the aldehyde (II), suggesting that the conversion of 32-alcohol (I) into 32-aldehyde (II) does not occur through a conventional alcohol dehydrogenase type of reaction.

Stereospecific oxidation at C-32. Expt. 1 in Table 4 shows that the metabolism of the [32-^{3}H]Δ^7-3,32-diol resulted in an unequal distribution of radioactivity in the two radioactive products, 46% being associated with water and 54% with formic acid. One possible explanation for this unequal distribution was that in the chemical synthesis the reduction of the aldehyde by NaB^3H_4 had occurred with partial stereospecificity, giving rise to the 32-alcohol containing ^{3}H predominantly in one of the two orientations. Such asymmetry would be retained during the subsequent stereospecific enzymic oxidations. Such a phenomenon was originally observed during work on the removal of the 19-methyl group in oestrogen biosynthesis (Skinner & Akhtar, 1969; also see Arigoni *et al.*, 1975). For convenience we shall refer to the Δ^7-3,32-diol made by the reaction:

$$-CH{=}O+NaB^3H_4 \rightarrow -CH^3HOH$$

as the A-diol. Consequently the enantiomeric B-diol was prepared by the reaction:

$$-C^3H{=}O+NaBH_4 \rightarrow C^3HHOH$$

When quantitatively metabolized by the microsomal fraction, the B-diol released more radioactivity in H_2O (67%) than in formic acid (33%). The results in Table 4 thus show that the ^{3}H at C-32 has predomin-

Table 4. *Stereospecific oxidation at C-32 of* [32-*A*-^{3}H]- *or* [32-^{3}H-*B*]-*lanost-7-ene-3β,32-diol*

Either [32-A-^{3}H]lanost-7-ene-3β-32-diol (A-diol; 16μg; 201600 d.p.m.) or [32-^{3}H-B]lanost-7-ene-3β-32-diol (B-diol; 16μg; 103800 d.p.m.) was incubated for 60min with washed microsomal fraction (5.0ml). The NADPH was regenerated and products were isolated as described in Table 1.

Expt. no.	[32-^{3}H]3,32-Diol prepared as:	^{3}H in acid-volatile distillate (% of original radioactivity)	^{3}H (% of acid-volatile radioactivity) in	
			Water	Formic acid
1	$-CH=O+NaB^3H_4$ ↓ A-diol	93.5	46	54
2	$-C^3H=O+NaBH_4$ ↓ B-diol	97.0	67	33

antly opposite stereochemical orientations in the two diols, and in addition they established that, during the metabolism of the Δ^7-3,32-diol, the hydrogen atoms at C-32 are biologically distinguished, the one with the orientation B being removed as a proton and the other with orientation A being ultimately released with formic acid.

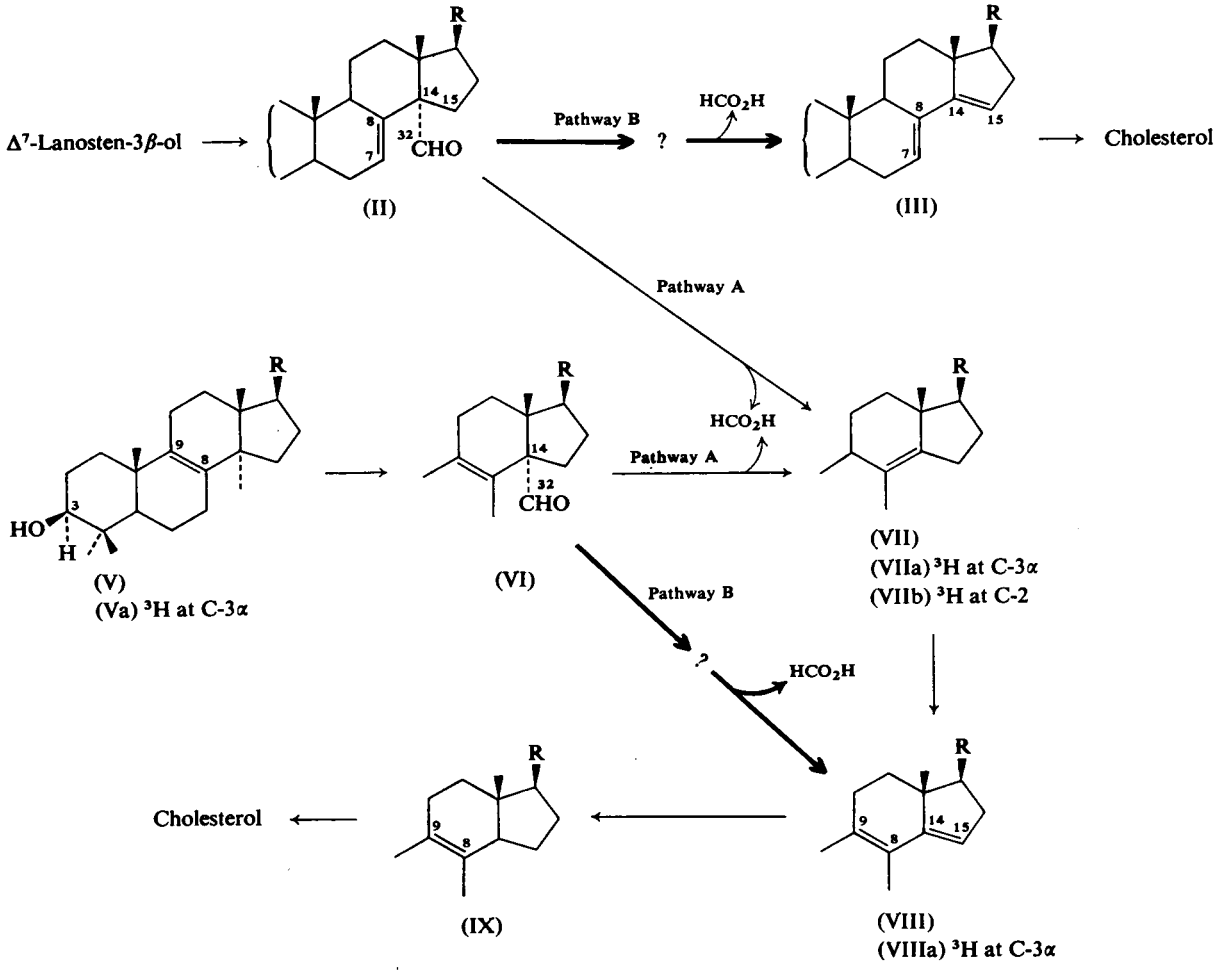
Characterization of the 32-*nor steroid*

Having established the chemical nature of some of the enzymic processes that occur at C-32 before the cleavage of the C-14–C-32 bond, our next problem was to identify the steroid produced after the bond scission. Additional background information pertinent in this connexion includes the knowledge that the removal of a 15α-hydrogen atom from lanosterol (V, Scheme 4) is intimately linked to the loss of C-32 (Canonica *et al.*, 1968*a*,*b*; Gibbons *et al.* 1968; Akhtar *et al.*, 1968, 1969*a*; Goodwin, 1971, and references cited therein), and also that a 8,14-diene system of type (VIII) is one of the early products of demethylation (Watkinson & Akhtar, 1968; Watkinson *et al.*, 1971; Fiecchi *et al.*, 1969). These features have been reconciled in terms of two alternative mechanistic proposals (Fiecchi *et al.*, 1972; Akhtar *et al.*, 1972; Watkinson *et al.*, 1971; Spike *et al.*, 1974, and references cited therein; Schroepfer *et al.*, 1972; Goodwin, 1971), which in the light of the new information that the C-32 is removed as formic acid and not as CO_2 may be presented in modified forms as shown in Scheme 4. Pathway A assumes that activation for the cleavage of the C-14–C-32 bond of the compound (VI) is provided through protonation of the double bond by a group on the enzyme, to give an electron-deficient centre at C-8 which facilitates deformylation to produce the $\Delta^{8(14)}$-steroid (VII). It is argued that the $\Delta^{8(14)}$-steroid (VII) cannot be isomerized directly to the $\Delta^{8(9)}$-steroid (IX) and that the conversion may occur indirectly by a two-step process involving first a desaturation reaction giving the 8,14-diene (VIII) and then the reduction of the 14,15 double bond (VIII → IX). An important point to remember is that, according to the basic tenets of the pathway A, the demethylation of a 14α-methyl Δ^7-steroid will also produce the $\Delta^{8(14)}$-steroid (14α-methyl steroid → II → VII), and the subsequent fate of the latter therefore must be the same, whether it originates from a $\Delta^{7(8)}$ or $\Delta^{8(9)}$ precursor, consequently yielding the 8,14-diene (VIII) as a common product.

The pathway B assumes that the loss of C-32 and the formation of a diene are closely linked processes. Furthermore, according to this hypothesis the demethylation of $\Delta^{7(8)}$- and $\Delta^{8(9)}$-steroids will result in the formation of 7,14- and 8,14-dienes respectively (Scheme 4). The salient features of these two pathways are experimentally evaluated below.

Is the $\Delta^{8(14)}$*-sterol a product of the metabolism of dihydrolanosterol?* The postulated pathway A (Scheme 4) envisages the $\Delta^{8(14)}$-sterol (VII) as an intermediate product of the metabolism of dihydrolanosterol (V). An attempt was therefore made to trap the $\Delta^{8(14)}$-sterol (VII) from the metabolism of [3α-^{3}H]dihydrolanosterol (Va). In view of the similar chromatographic properties of dihydrolanosterol (V) and the $\Delta^{8(14)}$-sterol (VII), the separation of these two compounds was performed after a chemical modification. The material from the band on t.l.c. that should contain the $\Delta^{8(14)}$-sterol (VII) and dihydrolanosterol (V) was acetylated and oxidized with 3-chloroperbenzoic acid, where the $\Delta^{8(14)}$-sterol acetate gave a unique epoxidation product of R_F 0.22 (see the Experimental section), whereas the oxidation product of dihydrolanosterol acetate did not run in this region. The failure to detect any radioactivity with the characteristic epoxidation product of the $\Delta^{8(14)}$-sterol (VII) (Table 5) is convincing evidence that this $\Delta^{8(14)}$-sterol (VII) is not a product of the metabolism of dihydrolanosterol (V). In a parallel control incubation the 8,14-diene (VIII) was trapped from dihydrolanosterol (V) in good yield (Table 5).

Experiments to establish whether the $\Delta^{8(14)}$*-sterol*



Scheme 4. *Two hypothetical pathways, A (→) and B(➔), for the cleavage of the C-14–C-32 bond of* Δ^7- *and* Δ^8-*steroids*

Table 5. *Experiments to establish whether the* $\Delta^{8(14)}$-*sterol (VII) is a direct precursor of the* 8,14-*diene (VIII) according to the sequence predicted by pathway A (Scheme* 4)

Sterols were aerobically incubated with rat liver homogenate (10.0ml) for 90min. The $\Delta^{8,(14)}$-sterol (VII) was isolated as a characteristic epoxidation product and the 8,14-diene (VIII) was isolated by t.l.c. as described in the Experimental section.

Substrate	Additions	Radioactivity (% of original) With 8,14-diene	With $\Delta^{8(14)}$
[3α-^{3}H]Dihydrolanosterol (Va) (100μg; 1200000 c.p.m.)	8,14-Diene (VIII) (0.5mg)	22	<1
[3α-^{3}H]Dihydrolanosterol (Va) (100μg; 1200000 c.p.m.)	8,14-Diene (VIII) (0.5mg) + $\Delta^{8(14)}$-Sterol (VII) (0.5mg)	21	<1
[3α-^{3}H]$\Delta^{8(14)}$-Sterol (VIIa) (100μg; 911500 c.p.m.)	8,14-Diene (VIII) (0.5mg)	0.9	—

Table 6. *Attempt to trap the $\Delta^{8(14)}$-sterol (VII) or the 7,14-diene (III) during the metabolism of the Δ^7-3,32-diol (I)*
The [3α-^{3}H]Δ^7-3,32-diol (Ib) (14μg; 568000 c.p.m.) was aerobically incubated with rat liver homogenate (5.0ml) for 120min and the sterols were isolated and characterized as described in the Experimental section.

Trap	^{3}H (% of original radioactivity) Associated with 7,14-diene (III)	Associated with $\Delta^{8(14)}$-sterol (VII)
$\Delta^{8(14)}$-Sterol (VII) (1.0mg)	—	<0.1
7,14-Diene (III) (1.0mg)	19	—
None	4	<0.1

(VII) is a precursor of the 8,14-diene (VIII) as predicted by pathway A (Scheme 4). Two samples of [3α-^{3}H]-dihydrolanosterol (Va) were incubated in the presence of a trap of the 8,14-diene (VIII); one of the experiments in addition contained the $\Delta^{8(14)}$-sterol (VII). As indicated in Table 5, the presence of the $\Delta^{8(14)}$-sterol had no significant effect on the amount of radioactivity trapped by the 8,14-diene (VIII). If the $\Delta^{8(14)}$-sterol (VII) is the direct precursor of the 8,14-diene (VIII), as predicted by pathway A (Scheme 4), then the presence of the unlabelled $\Delta^{8(14)}$-sterol (VII) during biosynthesis would be expected to diminish the incorporation of radioactivity from [3α-^{3}H]dihydrolanosterol (Va) into the 8,14-diene (VIII) through pool dilution. This was not the case.

The postulated pathway A (Scheme 4) also requires the $\Delta^{8(14)}$-sterol (VII) to be a better precursor of the 8,14-diene (VIII) than is dihydrolanosterol (V). However, the incubation of the [3α-^{3}H]$\Delta^{8(14)}$-sterol (VIIa) in the presence of a trap of the 8,14-diene (VIII) revealed that less than 1% of the original radioactivity was associated with the 8,14-diene trap, compared with 22% trapped into this diene (VIII) from [3α-^{3}H]dihydrolanosterol (Va) in a parallel experiment (Table 5).

Comparative study of the metabolism of the $\Delta^{8(14)}$-sterol (VII) and dihydrolanosterol (V) to cholesterol. Our failure to demonstrate a convincing intermediary role for the $\Delta^{8(14)}$-sterol (VII) led to a re-examination of a previous report (Fried *et al.*, 1968) that claimed that the $\Delta^{8(14)}$-sterol (VII) was at least as good a precursor of cholesterol as was dihydrolanosterol (V). Samples (100μg) of both [2-^{3}H]dihydrolanosterol and the [2-^{3}H]$\Delta^{8(14)}$-sterol were incubated with rat liver homogenate and the biosynthesized cholesterol was isolated as the dibromide. Under our conditions 15μg and 2.5μg of the respective precursors were converted into cholesterol, thus suggesting that the $\Delta^{8(14)}$-sterol (VII) was only one-sixth as good a precursor of cholesterol as was dihydrolanosterol (V). These findings have subsequently been confirmed [compare Alexander *et al.* (1971) with Fiecchi *et al.* (1972) and Gibbons (1974)].

Isolation of the 7,14-diene (III) as a product of the metabolism of the Δ^7-3,32-diol and exclusion of the $\Delta^{8(14)}$-sterol (VII) from an intermediary role

The most convincing evidence in support of an intermediary role for the $\Delta^{8(14)}$-sterol (VII) was a previous report in which it was claimed that an impressive quantity of this $\Delta^{8(14)}$-sterol accumulated during the metabolism of the Δ^7-3,32-diol (I) (Fried *et al.*, 1968). Our failure to find such an intermediary role for the $\Delta^{8(14)}$-sterol (VII) during the metabolism of the $\Delta^{8(9)}$-sterol (V) therefore led us to re-investigate this problem by using labelled precursors. The [3α-^{3}H]Δ^7-3,32-diol (Ib) was synthesized as described in the Experimental section, and a sample of this was aerobically incubated for 90min with rat liver homogenate (10.0ml) in the presence of the $\Delta^{8(14)}$-sterol (VII) (2.0mg). The extracted sterols were then subjected to extensive chromatographic separations (see the Experimental section), and the appropriate band was co-crystallized with authentic carrier $\Delta^{8(14)}$-sterol (VII). After five crystallizations, less than 0.2% of the original radioactivity in the incubation was found to be associated with the $\Delta^{8(14)}$-sterol (VII).

In a parallel experiment the [3α-^{3}H]Δ^7-3,32-diol (Ib) (22μg; 290000 c.p.m.) was incubated in the presence of 4,4-dimethylcholesta-7,14-dien-3β-ol (III) (2.0mg). Isolation of the 7,14-diene (III) band by t.l.c., followed by acetylation and co-crystallization with authentic carrier 4,4-dimethylcholesta-7,14-dien-3β-ol acetate, revealed that 19% of the radioactivity incubated was associated with the 7,14-diene (III) (Table 6). This was further confirmed by the retention of 93% of the radioactivity when the 4,4-dimethylcholesta-7,14-dien-3β-ol acetate was converted into 4,4-dimethylcholest-8(14)-ene-3β,7,15-triol (IV) by oxidation with perphthalic acid followed by alkaline hydrolysis.

It was necessary, however, to exclude the possibility that the ^{3}H retained in the triol (IV) did not originate from the 8,14-diene (structure of type VIII). Accordingly it was shown that when unlabelled 4,4-dimethylcholesta-7,14-dien-3β-ol acetate, in the presence of a trace amount of radioactive 4,4-

Table 7. *Cofactor requirements for the metabolism of the* Δ^7*-32-aldehyde (II)*

The [3α-^{3}H]Δ^7-32-aldehyde (IIb) (50μg; 1.54×10^6 d.p.m.) was aerobically incubated with washed microsomal fraction (5.0ml) for 30min in the presence of a trap of the 7,14-diene (III) (0.75mg). The concentration of NADPH in Expt. 1 corresponds to that of $NADP^+$ used in the regenerating system. The sterols were extracted with ether and isolated by t.l.c. as described in the Experimental section.

Expt. no.	Additions	Recovery of ether extract (%)	^{3}H (% of original radioactivity) associated with Δ^7-3,32-Diol (I)	Δ^7-32-Aldehyde (II)	7,14-Diene (III)+ 4,4-dimethylcholest-7-en-3β-ol
1	NADPH (0.5mM)	68	0	20.4	26.7*
2	NAD^+ (0.5mM)	100	0	89.8	2.9
3	$NADP^+$ (0.5mM)	98	0	90.8	2.3
4	None	100	0	92.2	2.1

* In this experiment about 17% of the recovered radioactivity was also associated with another compound which was tentatively characterized as 4β-methyl-4α-hydroxymethylcholest-7-en-3β-ol.

dimethylcholesta-8,14-dien-3β-ol acetate (structure of type VIII), was converted into the triol (IV) as above (Scheme 3) over 98% of the radioactivity from the 8,14-diene acetate was lost. This established that the oxidation product of the 8,14-diene acetate would not co-crystallize with the triol (IV) and confirmed that the radioactivity trapped in the above biosynthetic experiment was associated exclusively with the 7,14-diene (III). The greatly decreased accumulation of radioactivity in the 7,14-diene in the absence of a trap (Expt. 3, Table 6) suggests that under the normal conditions of cholesterol biosynthesis the activity of the succeeding enzyme of the pathway, $\Delta^{14(15)}$-reductase, is high. It was shown that the 7,14-diene (III) was also formed from the Δ^7-32-aldehyde (II), the next postulated intermediate of the pathway. An additional feature emerging from the data of Table 7 is that the formation of the 7,14-diene from the Δ^7-32-aldehyde was entirely dependent on the presence of NADPH and O_2. The cofactor requirement emphasizes why the accumulation of the Δ^7-32-aldehyde from the corresponding 32-alcohol in the experiments of Table 3 was achieved only in the presence of a limiting supply of NADPH. With excess NADPH, the aldehyde will be aerobically converted into 7,14-diene and thence into the $\Delta^{7(8)}$-steroid.

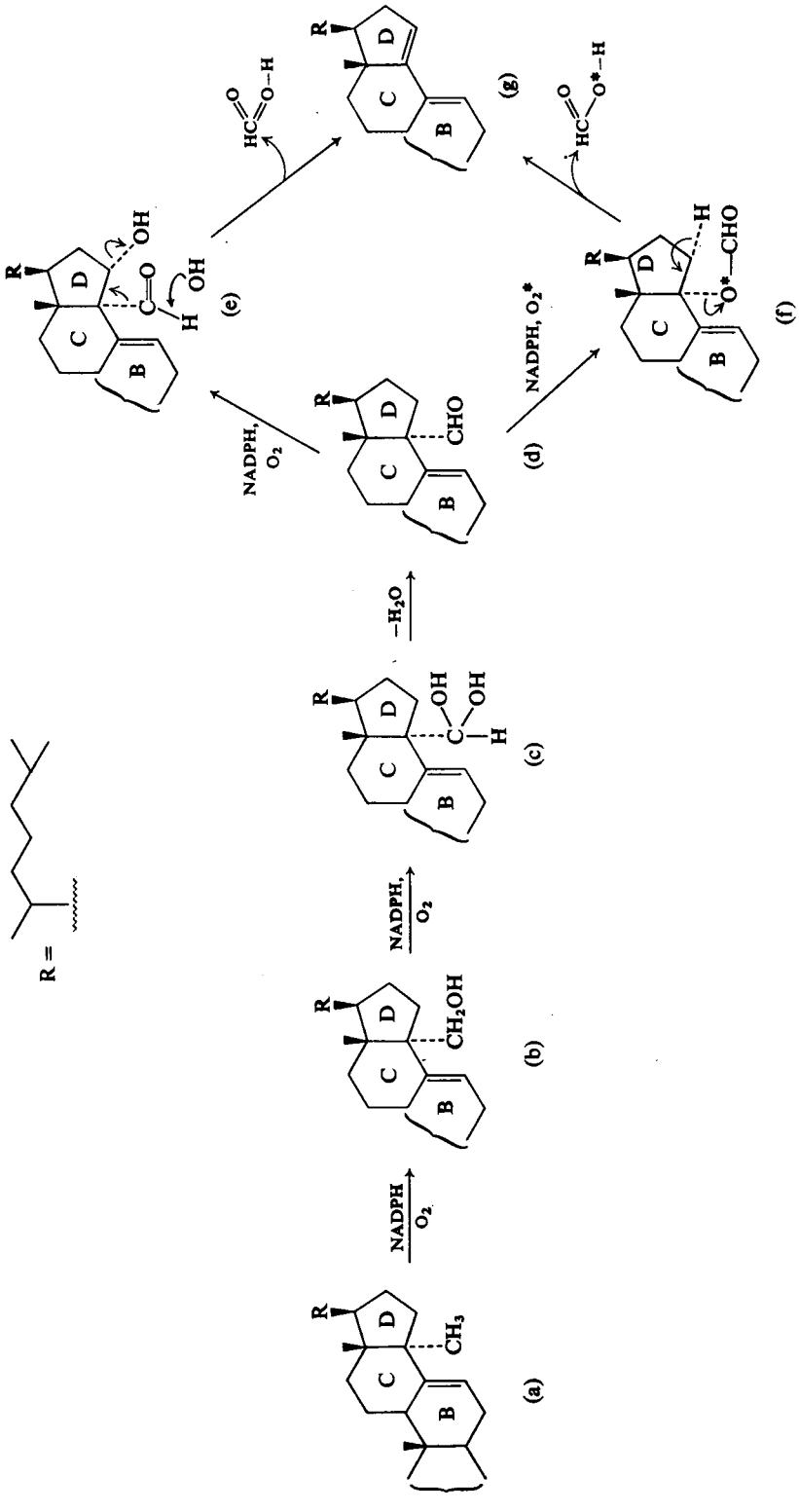
Relationship between the release of formic acid and the formation of the immediate products of 14-*demethylation*

Experiments were performed to establish the relationship between the release of formic acid and the formation of the immediate product of C-14 demethylation, 4,4-dimethylcholesta-7,14-diene-3β-ol (III). Either the [3α-^{3}H]- or [32-^{3}H]-Δ^7-3,32-diol (Ib or Ia) was incubated in the presence of a trap of the 7,14-diene (III) with washed microsomal fraction supplemented with NADPH generator. From the [32-^{3}H]Δ^7-3,32-diol (Ia) 47% of the original radioactivity was released into the acid-volatile fraction, whereas 32% of the radioactivity from the [3α-^{3}H]-Δ^7-3,32-diol (Ib) was found to be present in the 4,4-dimethylcholesta-7,14-dien-3β-ol (III) band on t.l.c. That over 90% of the radioactivity chromatographically coincident with the diene band was associated with 4,4-dimethylcholesta-7,14-diene-3β-ol (III) was shown by the conversion of its acetate in the presence of carrier into 4,4-dimethylcholesta-7,14-diene-3β,7,15-triol (IV) as described above (Scheme 3).

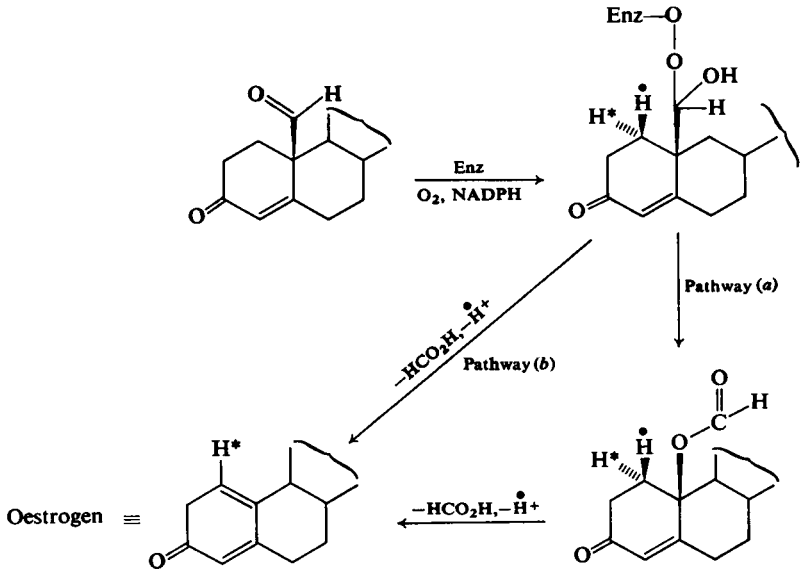
We have extended the types of experiments described in the present paper for lanost-7-ene-3β,32-diol to the corresponding compound in the Δ^8 series, lanost-8-ene-3β,32-diol, and have shown that the metabolism of the latter steroid also results in the release of C-32 as formic acid, but in this case, as expected, an 8,14-diene (V) system is formed (Freeman, 1977; Akhtar *et al.*, 1977).

Discussion

A detailed study of the mechanism of the removal of the 14α-methyl group in cholesterol biosynthesis has been carried out by using 32-oxygenated $\Delta^{7(8)}$-steroids. Cumulatively the results may be rationalized in terms of the sequence of Scheme 5, which allows the inference to be drawn that the biological transformation occurs through the intermediacy of a 32-hydroxy derivative (b, Scheme 5), although the formation of the latter compound under the conditions of cholesterol biosynthesis has not yet been demonstrated. The synthetic 32-hydroxy compound (of the type b) is efficiently metabolized by rat liver microsomal fraction, and in this process the C-32 is released as formic acid. The first step in the preceding



Scheme 5. *Postulated pathway for the 14α-demethylation*



Scheme 6. *Proposed mechanism for the removal of C-19 in oestrogen biosynthesis*

Pathways: (*a*) via Baeyer–Villiger process, (*b*) elimination via a concerted process. Whether in the first step it is an enzyme(Enz)–dioxygen or enzyme–monoxygen species that interacts with the substrate is a matter of conjecture at the present time. However, it is merely for the sake of mechanistic simplicity that an enzyme–dioxygen species has been used above.

conversion is the oxidation of the 32-alcohol (b) to the aldehyde (d). This does not occur through the conventional alcohol dehydrogenase type of reaction, but proceeds by a novel process requiring NADPH and molecular oxygen. Since NADPH and oxygen are required for a hydroxylation reaction of the type:

$$RH+O_2+NADPH \rightarrow R\text{–}OH+NADP^+ + H_2O$$

the conversion of 32-alcohol (b) into 32-aldehyde (d) may also be mediated by such a process involving the *gem*-diol (c) as an intermediate.

The hydroxylation mechanism for the conversion of alcohols into carbonyl compounds also seems to operate in other demethylation reactions, such as the removal of 4α- and 4β-methyl groups in cholesterol biosynthesis and also the elimination of the 19-methyl group in the biosynthesis of oestrogens from androgens (Skinner & Akhtar, 1969; see also Arigoni *et al.*, 1975). The extensive use of this otherwise unusual and rare mechanism for the conversion alcohol → aldehyde → acids during demethylation could be rationalized by suggesting that a single enzyme participates in the initial hydroxylation of a methyl group as well as the subsequent oxidation of the alcohol so produced. When the carbon atom is removed as CO_2, as in C-4 demethylation, then the *gem*-diol or its equivalent may undergo a third hydroxylation to furnish the corresponding carboxylic acid derivative.

Returning to the loss of C-32, the further metabolism of the 32-aldehyde (d) results in the formation of the 7,14-diene (g). This observation, taken in conjunction with the fact that removal of C-32 from $\Delta^{8(9)}$-steroids exclusively gives the corresponding 8,14-dienes of type VIII (see Scheme 4), unambiguously eliminates the much-debated role of a $\Delta^{8(14)}$-steroid in the C-14–C-32 bond cleavage. If a $\Delta^{8(14)}$-steroid was involved in an intermediary role in such a process, the same diene would have been produced from 14α-methyl-$\Delta^{7(8)}$ as well as -$\Delta^{8(9)}$ precursors. Additional evidence, of the type presented in Table 5, against the involvement of a $\Delta^{8(14)}$-steroid as an intermediate on the major pathway for cholesterol biosynthesis has received independent and subsequent confirmation [compare Alexander *et al.* (1971) with Fiecchi *et al.* (1972) and Gibbons (1974)].

The formation of the 7,14-diene and the release of C-32 as formate from the 32-aldehyde (d) once again depends entirely on the availability of NADPH and O_2. Conventionally the participation of NADPH and O_2 in the metabolism of compound (d) may be rationalized by proposing another hydroxylation, now at C-15 to yield the hydroxy aldehyde (e), which by the mechanism shown in Scheme 5 is converted into the 7,14-diene, releasing formic acid.

A point to remember is that according to this hypothesis the formate eliminated in the process

would incorporate one atom of oxygen from the water of the medium. NADPH and O_2 are also required for several other oxygen-insertion reactions, such as epoxidations and Baeyer–Villiger oxidations. Thus another mechanism for the conversion of compound (d) into compound (g) could involve the intermediacy of a –O–formyl derivative (f), which furnishes the 7,14-diene through the loss of the formyl group from C-14 and a proton from C-15. According to this mechanism in the conversion of compound (d) into compound (g), one of the oxygen atoms of the formate would be derived from molecular oxygen. Owing to several technical difficulties, experiments with $^{18}O_2$ and $H_2{}^{18}O$ have not yet been performed to study the mechanism of the removal of C-32 in cholesterol biosynthesis. We have, however, recently (Akhtar *et al.*, 1976) drawn attention to the close chemical and enzymological similarities that exist between the removal of C-32 in cholesterol and C-19 in oestrogen biosyntheses. During studies on oestrogen biosynthesis it was shown that the elimination of C-19 from the 19-aldehyde occurred in a NADPH- and oxygen-dependent reaction and in this process one atom of oxygen from $^{18}O_2$ was incorporated into formic acid (Akhtar *et al.*, 1976). This result was interpreted to suggest that the cleavage of C-10–C-19 bond may occur by a Baeyer–Villiger type of process, for which two alternative mechanisms have been considered (Scheme 6). In view of this precedent, there is a strong possibility that mechanism(s) of the type suggested for the C-10–C-19 bond-cleavage step in oestrogen biosynthesis may also operate for the removal of C-32 in cholesterol biosynthesis (Akhtar *et al.*, 1976).

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