LANOSTEROL 14α-DEMETHYLASE. THE METABOLISM OF SOME POTENTIAL INTERMEDIATES BY CELL-FREE SYSTEMS FROM RAT LIVER

By

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Received February 10,1976

SUMMARY

A number of potential intermediates of lanosterol* 14α-demethylation have been synthesized for the first time and labelled with ^{3}H. A direct comparison of the rates of conversion of each of these materials to cholesterol and 5α-cholest-7-en-3β-ol by a cell-free system from rat liver has been made. Although 5α-lanost-8-en-3β,32-diol and 3β-hydroxy-5α-lanost-8-en-32-al were converted to C_{27} sterols at a greater rate than was 5α-lanost-8-en-3β-ol, the apparent K_m values were larger than those expected if these compounds were obligatory intermediates. 5α-Lanost-8-en-3β,15α-diol and 5α-lanost-8-en-3β,15β-diol were poorer precursors of cholesterol but each was extensively converted both to a more polar compound and to the corresponding 3β,15-diol diester.

Recent investigations have revealed the complexity of the process by which the 14α-methyl (C-32) group of lanosterol is removed during cholesterol biosynthesis. Some pertinent observations include the release of C-32 as formic acid (1), the stereospecific removal of the 15α-hydrogen (2,3) and the formation of a conjugated $\Delta^{8,14}$ dienol (4,5, VIII, Fig. 1). In addition, the requirement for cytochrome P-450 during 14α-demethylation (6,7) may result in an interaction between cholesterol biosynthesis and drug metabolism. However, the oxidative sequence and the nature of the oxidized intermediates which must be involved remain matters of speculation. One proposal involves a succession of three oxidative events, the first of which gives rise to a 14α-hydroxymethyl compound ((1), Sequence V $\longrightarrow$ I $\longrightarrow$ II $\longrightarrow$ VII $\longrightarrow$ VIII, Fig. 1). Alternatively, there is some evidence (8-10) which suggests that a

* Systematically, lanosterol is 5α-lanosta-8,24-dien-3β-ol, dihydrolanosterol is 5α-lanost-8-en-3β-ol and cholest-7-enol is 5α-cholest-7-en-3β-ol.

15-hydroxylation is the first step (Sequence V $\rightarrow$III (or IV) $\rightarrow$ VI $\rightarrow$VII $\rightarrow$VIII, Fig. 1) and facilitation of 4α-methyl group oxidation by the neighbouring 3-hydroxy function (11) lends credibility to such a pathway. Up to now, however, no direct comparison has been made of the ability of C-15 and C-32 functionalized lanostenols to fulfil the criteria for obligatory intermediacy. To this end we have synthesized the various oxidized lanost-8-enol derivatives required to determine which, if any, of the alternative pathways operates physiologically, and the present report describes their metabolism by a cell-free system of rat liver.

MATERIALS AND METHODS

Synthesis of 5α-[16-$^3H_2$]lanost-8-en-3β,15α-diol and 5α-[16-$^3H_2$]lanost-8-en-3β,15β-diol

3β-Hydroxy-5α-lanost-7-en-15-one (12) was labelled at position 16 by exchange with ^3H_2O (0.01 ml, 50 mCi) in dioxane solution for 5.5 h at 95° in the presence of sodium methoxide. Reduction of the recovered [16-$^3H_2$]-15-one with $LiAlH_4$, addition of non-radioactive material to each of the epimeric Δ^7-3β,15-diol products followed by acetylation, acid isomerization and hydrolysis (13) of each gave, after chromatographic purification, 5α-[16-$^3H_2$]-lanost-8-en-3β,15α-diol (5.97×10^7 d.p.m./μmole) and 5α-[16-$^3H_2$]lanost-8-en-3β,15β-diol (3.48×10^7 d.p.m./μmole).

Synthesis of 5α-[2-$^3H_2$]lanost-8-en-3β,32-diol and 3β-hydroxy-5α-[2-$^3H_2$]-lanost-8-en-32-al

5α-Lanostan-3β,7α-diol-3-acetate (m.p., 212°; $[\alpha]_D^{25}$ + 14.6*) was synthesized by the method of Barton and Thomas (14), who quote m.p., 205-206°; $[\alpha]_D^{25}$ + 14°. Treatment of this material with lead tetraacetate (15,16) followed by alumina chromatography of the crude reaction product gave 3β-acetoxy-5α-lanostan-7α,32-oxide, m.p., 195-197°; $[\alpha]_D^{25}$ + 25° (reported: m.p., 202-204°; $[\alpha]_D^{25}$ + 25° (16)). Treatment of the cyclic ether with pyridinium chloride and acetic anhydride (16,17) gave three products which, after hydrolysis, were separable by column or t.l.c. The product of intermediate polarity was formed in the largest amount and corresponded to 5α-lanost-7-en-3β,32-diol, m.p., 208-210°; $[\alpha]_D^{25}$ + 10.7° (reported: m.p., 204-205°; $[\alpha]_D^{25}$ + 10° (18)). A minor (19 %) product had the greatest polarity, and after several recrystallizations from aqueous acetone had m.p., 174-175°; $[\alpha]_D^{25}$ + 52.1°. The chemical and spectroscopic properties of this material were consistent with the structure 5α-lanost-8-en-3β,32-diol. Jones oxidation of this material (19) gave 5α-lanost-8-en-3,32-dione, m.p., 120-122°; $[\alpha]_D^{25}$ - 229°. This material was labelled by exchange with ^3H_2O (0.01 ml; 50 mCi) in a sealed tube under basic conditions at 70° C for 3 h in an atmosphere of nitrogen. Recovery of the labelled material followed by partial reduction (2.5 equiva-

* Measurements of specific rotation were made in chloroform (c = 1).

lents KBH_4 in 90 % aqueous dioxan (1.0 ml) for 15 min at 25°) of a portion gave, after t.l.c., 3β-hydroxy-5α-[2-$^3H_2$]lanost-8-en-32-al (1.428 x 10^7 d.p.m./μmole) identical in all respects with an unlabelled sample of m.p. 160-161°; $[\alpha]_D^{25}$ - 130.5°. Complete reduction of another portion of the [2-$^3H_2$]-3,32-dione (identical conditions to the above except that the reaction time was 20 h) gave 5α-[2-$^3H_2$]lanost-8-en-3β,32-diol (1.400 x 10^7 d.p.m./μmole), identical spectroscopically and chromatographically with the non-radioactive material prepared above.

Preparation of rat-liver subcellular fractions and incubation procedures

Rats (male Wistars weighing 150-200 g) were killed at 08.30 h on the day of the experiment. After excision of the liver, subcellular (S_{10}) fractions were prepared as described previously (6). Procedures for emulsification of substrates, incubation and total lipid extraction have been previously described (6,7). T.l.c. of the total lipid fraction on silicagel H using chloroform as the mobile phase was routinely used for the initial separation of the labelled C_{27} sterol mixture from its precursors. In this system (System 1) the R_s (cholesterol = 1) values of the substrates were:- I, 0.30; II, 1.00; III, 0.88; IV, 1.04; V and VIII, 1.30. Because of the similar polarities of cholesterol and two of its substrates, II and IV, the C_{27} sterol mixture was, in practice, severely contaminated with these materials after chromatography in system 1. Radioactivity due to the precursor sterol was removed in these instances by acetylation of the mixture followed by t.l.c. on silicagel H, using a mixture of chloroform and hexane (75:25, v/v) as the mobile phase. This is System 2. Finally, cholesterol was separated from cholest-7-enol as the acetates by t.l.c. on silver nitrate-impregnated alumina using a mixture of toluene and hexane (25:75, v/v) as the mobile phase (System 3 (20)).

RESULTS

Conversion of the 5α-lanost-8-en-3β,15-diols to C_{27} sterols

5α-[16-$^3H_2$]Lanost-8-en-3β,15α-diol (71.2 nmoles; 4.250 x 10^6 d.p.m.) and 5α-[16-$^3H_2$]lanost-8-en-3β,15β-diol (91.9 nmoles; 3.201 x 10^6 d.p.m.) were incubated independently with an S_{10} fraction of rat liver (1.9 ml; 42.2 mg protein) in a total volume of 2.5 ml for 1 h at 37° C. With each substrate, a control was set up in which enzymic activity was destroyed immediately after addition of the substrate, by the addition of a mixture of chloroform and methanol (2:1, v/v). Fractions containing labelled cholesterol and 5α-cholest-7-enol were isolated as the acetates and assayed for radioactivity. These compounds were formed in 0.31 % (98 ng) and 0.15 % (47 ng) yield respectively when 5α-lanost-8-en-3β,15α-diol was the precursor and in 2.11 % (860 ng) and 0.71 % (290 ng) yield when the 3β,15β-diol was used. These figures are an average of the results obtained from duplicate

incubations. The constancy of specific radioactivity during four recrystallizations (after addition of carrier) of each of the C_{27} steryl acetates labelled from the two 3β,15-diols provided proof of their radiochemical identity and purity. The specific radioactivities of the cholesteryl acetate and cholest-7-enyl acetate isolated from the "control" incubations with both substrates fell to zero after two recrystallizations.

Further examination of other radioactive fractions obtained after chromatography in System 1 revealed that a large proportion of both the 3β,15α-diol (27 %) and of the 3β,15β-diol (18 %) had been converted to a more polar compound (R_F 0.00 - R_F 0.12). The control incubations contained little or no radioactivity in this area of the plate. In addition, both diols were converted to compounds with the chromatographic properties of the 3β,15α- and the 3β,15β-diol diesters (2.7 % and 16.0 % respectively). Alkaline hydrolysis in each case gave rise to the original diol.

Conversion of 5α-lanost-8-en-3β,32-diol and 3β-hydroxy-5α-lanost-8-en-32-al to C_{27} sterols

5α-[2-$^3H_2$]Lanost-8-en-3β,32-diol (90.1 nmoles; 1.261 x 10^6 d.p.m.) and 3β-hydroxy-5α-[2-$^3H_2$]lanost-8-en-32-al (90.5 nmoles; 1.292 x 10^6 d.p.m.) were incubated independently with an S_{10} fraction of rat liver (1.0 ml; 19.8 mg protein) in a total volume of 2.5 ml for 45 min at 37° C. Again, for each substrate, non-incubated controls were carried out. The former substrate was converted to cholesterol and cholest-7-enol, isolated as the acetates as described above, in yields of 1.99 % (790 ng) and 2.38 % (950 ng) respectively, whilst the 3β-hydroxy-32-aldehyde resulted in a conversion of 2.35 % (940 ng) and 2.18 % (870 ng) respectively. There was very little radioactivity associated with the C_{27} steryl acetate fractions isolated from the control incubations. Again, the radiochemical purity of each of the labelled C_{27} steryl acetates from both types of incubation was established by addition of carrier material followed by crystallization to constant specific radioactivity. In all cases the specific radioactivities remained constant

during three recrystallizations at values similar to the corresponding initial calculated values. Similar incubations containing twice the above concentrations of each substrate were conducted and yielded identical qualitative results. In all these incubations, radioactive substances other than C_{27} sterols were formed in relatively very small quantities.

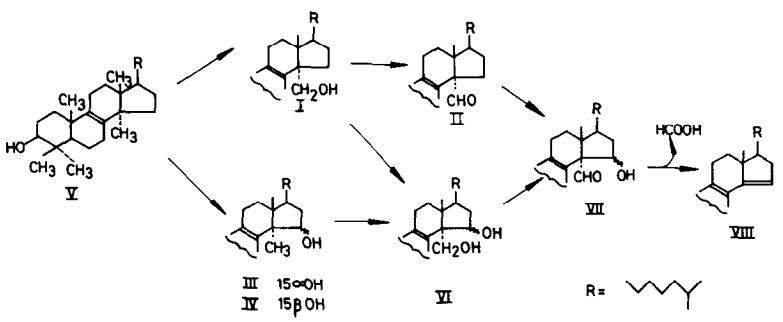

FIG.1 POSSIBLE BIOSYNTHETIC SEQUENCES INVOLVED IN 14α-DEMETHYLATION

Kinetics of conversion of the oxygenated lanost-8-enols to cholest-7-enol

An S_{10} fraction from rat liver was incubated with three different concentrations of each of the oxygenated lanost-8-enol derivatives. In addition, similar incubations were conducted with a range of concentrations of dihydro-[^{14}C]lanosterol (Structure I, Fig. 1) and 4,4-dimethyl-[2-$^{3}H_2$]-cholesta-8,14-dienol (Structure VIII, Fig. 1) in order to make direct comparisons with the kinetics of established intermediates of cholesterol biosynthesis. Cholesterol and cholest-7-enol were isolated as the acetates as described above. Lineweaver-Burk plots were constructed for the conversion of each substrate to cholest-7-enol and from these the values for apparent K_m and V_{max} were calculated. These values are summarized in Table 1. Unexpectedly, the 3β,15β-diol at a concentration of 144 μM resulted in a decreased rate of C_{27} sterol synthesis compared with that observed at lower concentrations of this substrate, and this phenomenon occurred again in subsequent

Table 1. Kinetics of conversion of some known and potential 4,4-dimethyl sterol precursors to cholesterol and 5α-cholest-7-enol

Substrate	Concentration range (μMolar)	Conversion (nmoles)* to:-		Apparent K_m (M) (Substrate → cholest-7-enol	V_{max} (nmoles/mg protein/45 min
		Cholesterol	Cholest-7-enol		
Dihydrolanosterol	9.4, 18.7, 93.5	1.203	2.411	5.00×10^{-5} (0.999)**	0.238
4,4-Dimethylcholesta-8,14-dienol	9.7, 19.4, 77.7	1.374	2.711	8.85×10^{-6} (0.980)	0.205
5α-Lanost-8-en-3β,15α-diol	18.0, 54.0, 144.0	0.184	0.390	2.00×10^{-3} (0.980)	0.230
5α-Lanost-8-en-3β,32-diol	36.0, 72.0, 144.0	2.835	7.689	2.33×10^{-4} (0.973)	1.212
3β-Hydroxy-5α-lanost-8-en-32-al	21.7, 54.3, 108.6	3.846	7.572	2.22×10^{-4} (0.950)	1.258

Incubations were carried out at 37° for 45 min with S_{10} fraction (0.8 ml; 15.0 mg protein); 30 mM nicotinamide; 10 mM GSH; 1 mM EDTA; 12 mM glucose 6-phosphate; 2 mM $NADP^+$; 0.8 mM NAD^+; 1.3 mM NADH; 1.3 mM ATP; 4 mM $MgCl_2$; 1 unit of glucose 6-phosphate dehydrogenase (1 μmole of NADPH generated/min) and 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 1.25 ml. Incubations were started by the addition of the substrate suspension in potassium phosphate buffer, pH 7.4 (0.1 ml) containing Tween 80 (1.25 mg). After incubation, in order to adjust for losses during the subsequent chromatographic procedures, [1α,2α-^{3}H]cholesterol (24,800 d.p.m.) was added to flasks containing dihydrolanosterol and [4-^{14}C]cholesterol (6336 d.p.m.) to all the rest. The reported values above take account of all manipulative losses.

* Conversion to C_{27} sterols at the highest concentration of substrate. ** Correlation coefficient.

experiments. For this reason it was impossible to calculate K_m and V_{max} at the concentrations used, and these values have been omitted from Table 1.

DISCUSSION

In the present work we have undertaken a direct comparison of kinetic parameters observed during the metabolism of various possible oxygenated lanosterol intermediates of cholesterol biosynthesis. During the conversion of lanosterol to cholesterol, nuclear double-bond rearrangement from Δ^8 to Δ^7 does not occur to any significant degree until the 14α-methyl group has been removed (21). Any oxidized intermediate involved in 14α-demethylation will therefore contain a Δ^8 bond, and the use of any other type of substrate may introduce further complications into an already complex problem. We have gone to some lengths, therefore, to ensure that the substrates used in this study are as closely related as possible to those structures which the demethylase enzymes would normally encounter. Although all the compounds tested were metabolized, the rates of conversion to C_{27} sterols varied widely. Both the epimeric 3β,15-diols were converted to a small extent, the 15β-epimer being a better precursor than the 15α at low concentrations of substrate. This result is at variance with reports (9,22) showing that 14α-methylcholest-7-en-3β,15α-diol is not converted to cholesterol and may justify the present policy of utilizing as substrates compounds more closely related to probable physiological intermediates. The low rates of conversion of 3β,15-diols to cholesterol may argue against the participation of these compounds as obligatory intermediates in cholesterol biosynthesis from dihydrolanosterol. However, their high rates of conversion to more polar compounds and to the 3,15-diol-diesters might suggest that compounds of this type are normally encountered by liver enzymes and enter into some biosynthetic pathway. In particular, the high rate of esterification of the hindered 15β-hydroxyl group is of interest.

Although the rates of conversion of the C-32 oxygenated compounds to C_{27}

sterols were high, the apparent K_m values were substantially larger than those observed with dihydrolanosterol or 4,4-dimethylcholesta-8,14-dienol, indicating a relatively low enzymic affinity for these types of substrate. The very high enzymic affinity for the obligatory cholesterol precursor, 4,4-dimethylcholesta-8,14-dienol, is consistent with its non-accumulation in tissues undergoing cholesterol synthesis under normal conditions.

The process of 14α-demethylation is strikingly similar to the incompletely understood mechanism by which the C-19 methyl group is removed during conversion of the androgens to the oestrogens, and it would appear that nature has elaborated the same general mechanism for the removal of steroidal tertiary methyl groups. It is likely that a solution to either of these important and intricate problems will also provide an answer to the other.

REFERENCES

1. Alexander, K., Akhtar, M., Boar, R.B., McGhie, J.F. and Barton, D.H.R. (1972) J. Chem. Soc. Chem. Commun., pp. 383-385.
2. Gibbons, G.F., Goad, L.J. and Goodwin, T.W. (1968) J. Chem. Soc. Chem. Commun., pp. 1458-1460.
3. Canonica, L., Fiecchi, A., Galli-Kienle, M., Scala, A., Galli, G., Grossi Paoletti, E. and Paoletti, R. (1968) Steroids 12, 445.
4. Fiecchi, A., Galli Kienle, M., Scala, A., Galli, G., Grossi Paoletti, E., Cattabeni, F. and Paoletti, R. (1972) Proc. Roy. Soc. Lond., Ser. B, 180, 147-165.
5. Akhtar, M., Wilton, D.C., Watkinson, I.A. and Rahimtula, A.D. (1972) Proc. Roy. Soc., Lond., Ser. B, 180, 167-177.
6. Gibbons, G.F. and Mitropoulos, K.A. (1973) Biochem. J. 132, 439-448.
7. Gibbons, G.F. and Mitropoulos, K.A. (1973) Europ. J. Biochem. 40, 267-273.
8. Huntoon, S. and Schroepfer, G.J., Jr. (1970) Biochem. Biophys. Res. Commun. 40, 476-480.
9. Martin, J.A., Huntoon, S. and Schroepfer, G.J., Jr. (1970) Biochem. Biophys. Res. Commun. 39, 1170-1174.
10. Gibbons, G.F. and Mitropoulos, K.A. (1975) Biochim. Biophys. Acta 380, 270-281.
11. Swindell, A.C. and Gaylor, J.L. (1968) J. Biol. Chem. 243, 5546-5555.
12. Woodward, R.B., Patchett, A.A., Barton, D.H.R., Ives, D.A.J. and Kelly, R.B. (1957) J. Chem. Soc., pp. 1131-1144.
13. Gibbons, G.F. and Ramananda, K. (1975) J. Chem. Soc. Chem. Commun., pp. 213-214.
14. Barton, D.H.R. and Thomas, B.R. (1953) J. Chem. Soc., pp. 1842-1847.
15. Tadanier, J. (1963) J. Org. Chem. 28, 1744-1751.
16. Fried, J., Brown, J.W. and Borkenhagen, L. (1965) Tetrahedron Lett., pp. 2499-2504.
17. Wall, M.E., Kenney, H.E. and Rothman, E.S. (1955) J. Amer. Chem. Soc. 77, 5665-5668.

18. Fried, J. and Brown, J.W. (1966) Tetrahedron Lett., pp. 1677-1680.
19. Djerassi, C., Engle, R.R. and Bowers, A. (1956) J. Org. Chem. 21, 1547-1549.
20. Gibbons, G.F. (1974) Biochem. J. 144, 59-68.
21. Gaylor, J.L., Delwiche, C.V. and Swindell, A.C. (1966) Steroids 8, 353-363.
22. Spike, T.E., Wang, A.H.-J., Paul, I.C. and Schroepfer, G.J., Jr. (1974) J. Chem. Soc. Chem. Commun., pp. 477-478.