The Pathway for the Conversion of Dihydroagnosterol into Cholesterol in Rat Liver

By I. ANTHONY F. TAVARES, KENNETH A. MUNDAY and DAVID C. WILTON

*Department of Physiology and Biochemistry, University of Southampton,
Southampton SO9 3TU, U.K.*

(Received 1 November 1976)

Dihydroagnosterol is demethylated by a rat liver homogenate to give 4,4'-dimethylcholesta-7,9-dienol and then cholesta-7,9-dienol. The cholesta-7,9-dienol is isomerized to cholesta-8,14-dienol, which is converted into cholesterol by the normal pathway.

Agnosterol and dihydroagnosterol (I), two naturally occurring mammalian sterols, were first identified in sheep wool fat. The biosynthesis of both compounds from [^{14}C]acetate was studied in rat liver by Clayton & Bloch (1956) and the efficient conversion of dihydroagnosterol into cholesterol by rat liver homogenate has been observed by Rahimtula *et al.* (1969).

The precise sequence for the conversion of dihydroagnosterol (I) into cholesterol (VIII) has still to be determined. An obvious pathway would involve the initial reduction of the $\Delta^{9(11)}$ -double bond to give Δ^7 -lanosterol (III), which may then be metabolized to cholesterol (VIII) by way of 4,4'-dimethylcholesta-7,14-dienol. However, there is no conversion of dihydroagnosterol (I) into Δ^7 -dihydrolanosterol (III) in the presence of NADPH under anaerobic conditions (Akhtar *et al.*, 1972). Similarly, cholesta-7,9-dienol (VI) is not initially reduced to Δ^7 -cholesterol, but is first rearranged to the cholesta-8,14-dienol (VII), which may then be converted into cholesterol in the normal manner (Akhtar *et al.*, 1972) (Scheme 1). In view of the above observations it seems probable that dihydroagnosterol (I) is demethylated at the C-14 position with the $\Delta^{7,9}$ -diene system still intact. This could be followed by isomerization of the resulting 4,4'-dimethylcholesta-7,9-dienol (II) to the 4,4'-dimethylcholesta-8,14-dienol (V). Alternatively, the C-4-dimethyl groups could be removed next to give cholesta-7,9-dienol (VI), which is then isomerized to the $\Delta^{8,14}$ isomer and hence to cholesterol (VIII) (see Scheme 1). The pathway for the conversion of dihydroagnosterol (I) into cholesterol (VIII) was investigated in order to distinguish between these possibilities.

Experimental

Materials were obtained, radioactivity was measured and rat liver supernatants were prepared as

described by Bloxham *et al.* (1971). Incubations were carried out and processed and cholesterol was isolated and purified as described by Wilton *et al.* (1968). Incubations involving the 10000 g_{av} supernatant were fortified with glucose 6-phosphate (2mM) and glutathione (2mM). Chromatographic analysis of the steryl acetates were undertaken by the methods of Rahimtula *et al.* (1969).

Dihydroagnosterol was prepared by the method of Birchenough & McGhie (1949). Dihydroagnosterol was oxidized with Jones reagent (Bowden *et al.*, 1946) and the derived 3-ketone was treated with sodium borotritide to give [3α - ^3H]dihydroagnosterol. The experimental details for the synthesis of ^3H -labelled compounds by the above sequence are described by Dewhurst & Akhtar (1967).

Preparation of 4,4'-dimethyl[2- ^3H]cholesta-7,9-dienol

4,4'-Dimethylcholesta-5,7-dienol prepared by the method of Gautschi & Bloch (1958) was reduced to 4,4'-dimethylcholesta-7-enol in the presence of Raney nickel and hydrogen (Watkinson *et al.*, 1971).

The 4,4'-dimethylcholesta-7,9-dienol was prepared from 4,4'-dimethylcholesta-7-enol on treatment with mercuric acetate by the method of Heusser *et al.* (1952) as described by Akhtar *et al.* (1972).

The 7,9-dienol was oxidized with Jones reagent (Bowden *et al.*, 1946). The derived 3-ketone was treated with methanolic 5% (w/v) KOH containing $^3\text{H}_2\text{O}$ (100mCi) overnight at 20°C. The product obtained was reduced with LiAlH_4 to give 4,4'-dimethyl[2- ^3H]cholesta-7,9-dienol.

Mass-spectral analysis in an AEI MS30 combined g.l.c.-mass spectrometer of the 4,4'-dimethylcholesta-7,9-dienyl acetate gave ions at m/e 454 (M^+), 439 ($M^+ - \text{CH}_3$), 394 ($M^+ - \text{CH}_3\text{CO}_2$), 379 ($M^+ - \text{CH}_3 - \text{CH}_3\text{CO}_2$; most intense ion), 281 (M^+ -side chain). The compound had a u.v.-absorption maximum at 243nm.

Scheme 1. Possible pathways for the conversion of dihydroagnosterol into cholesterol

Results

Utilization of [3 α -³H]dihydroagnoterol (I)

The conversion of lanosterol into cholesterol (VIII) involves removal of the 4 α -, 4 β - and 14 α -methyl groups by a sequence involving oxygen and NADPH (Miller *et al.*, 1967; Alexander, 1974) in which it is the 14 α -methyl group that is the first to be removed (Gautschi & Bloch, 1958). It was predicted that the conversion of dihydroagnoterol (I) into cholesterol (VIII) would follow a similar pattern, involving an oxidative removal of the 14 α -methyl group to give 4,4'-dimethylcholesta-7,9-dienol (II) as shown in Scheme 1.

[3 α -³H]Dihydroagnoterol (I) (40 μ g; 2 $\times$ 10⁶ d.p.m.) was incubated with a 10000g_{av.} supernatant of rat liver homogenate anaerobically under N₂ or aerobically in air. Under anaerobic conditions, essentially all the radioactivity incubated was recovered unchanged in dihydroagnoterol (I), the starting substrate. During aerobic incubations, 4,4'-dimethyl-[3 α -³H]cholesta-7,9-dienol (II) accumulated in about 16% yield.

Formation of 4,4'-dimethyl[3 α -³H]cholesta-7,9-dienol (II) from [3 α -³H]dihydroagnoterol (I)

[3 α -³H]Dihydroagnoterol (I) was incubated with a 10000g_{av.}-supernatant enzyme system in the presence of a 'trap' of nonradioactive 4,4'-dimethylcholesta-8,14-dienol (V). As shown in Table 1, 22.8% of the recovered radioactivity was associated with the 4,4'-dimethylcholesta-7,9-dienyl acetate band, whereas only 2.6% was associated with the 4,4'-dimethylcholesta-8,14-dienyl acetate band. 4,4'-Dimethyl[3 α -³H]cholesta-7,9-dienyl acetate, isolated from the above experiment, was mixed with non-radioactive 4,4'-dimethylcholesta-7,9-dienyl acetate (9 mg) and recrystallized from cold methanol/diethyl ether, during which process there was complete retention of radioactivity (Table 1).

On the other hand, recrystallization of the 4,4'-dimethylcholesta-8,14-dienyl acetate band with non-radioactive 4,4'-dimethylcholesta-8,14-dienyl acetate (9 mg) resulted in loss of radioactivity with each successive recrystallization. Mass-spectral analysis confirmed that the biosynthetic 4,4'-dimethyl-[3 α -³H]cholesta-7,9-dienyl acetate had a molecular weight of 454 and a fragmentation pattern identical with that of chemically synthesized 4,4'-dimethylcholesta-7,9-dienyl acetate. These results indicate that there is an enzyme system present in rat liver which converts dihydroagnoterol (I) into 4,4'-dimethylcholesta-7,9-dienol (II). The precise mechanism for this transformation has yet to be determined.

The further metabolism of the 4,4'-dimethylcholesta-7,9-dienol (II) does not appear to involve

Table 1. Formation of 4,4'-dimethyl[3 α -³H]cholesta-7,9-dienol (II)

Aerobic incubations of [3 α -³H]dihydroagnoterol (I) (40 μ g; 2 $\times$ 10⁶ d.p.m.) were performed with a 10000g_{av.} supernatant of rat liver homogenate (5 ml) in the presence of unlabelled 4,4'-dimethylcholesta-8,14-dienol (V) (500 μ g). The sterols were extracted, purified by t.l.c., acetylated and separated by t.l.c. on silica gel/10% (w/w) AgNO₃. The fractions corresponding to the dimethyl-7,9-diene and -8,14-diene were each recrystallized three times after addition of the corresponding unlabelled steryl acetate.

Steryl acetate isolated from AgNO ₃ t.l.c.	10 ⁻³ $\times$ Total radioactivity (d.p.m.)	% of total acetylated extract
4,4'-Dimethylcholesta-8,14-dienyl acetate	163	2.6
4,4'-Dimethylcholesta-7,9-dienyl acetate	1390	23
Dihydroagnoterol acetate	3600	59
Steryl acetate	No. of recrystallization	10 ⁻² $\times$ Sp. radioactivity (d.p.m./mg)
4,4'-Dimethylcholesta-7,9-dienyl acetate	1	551
	2	571
	3	570
4,4'-Dimethylcholesta-8,14-dienyl acetate	1	160
	2	95
	3	51

an isomerase at this stage, as there was no significant trapping of radioactivity in 4,4'-dimethylcholesta-8,14-dienol (V).

Lack of conversion of 4,4'-dimethylcholesta-7,9-dienol (II) into 4,4'-dimethylcholesta-8,14-dienol (V)

That the 4,4'-dimethylcholesta-7,9-dienol (II) does not isomerize to 4,4'-dimethylcholesta-8,14-dienol (V) was proved conclusively by incubating 4,4'-dimethyl[2-³H]cholesta-7,9-dienol (II) (100 μ g; 5.5 $\times$ 10⁵ d.p.m.) anaerobically with rat liver microsomal fraction in the absence of NADPH. All the recovered radioactivity was still associated with the starting substrate dimethyl[2-³H]cholesta-7,9-dienol (II). Under similar conditions [2-³H]cholesta-7,9-dienol (VI) (100 μ g; 8.0 $\times$ 10⁵ d.p.m.) isomerized to [2-³H]cholesta-8,14-dienol (VII) in 30% yield.

Conversion of 4,4'-dimethyl[2-³H]cholesta-7,9-dienol (II) into [2-³H]cholesta-7,9-dienol (VI)

Incubation of 4,4'-dimethyl[2-³H]cholesta-7,9-dienol (II) with a 10000g_{av.} supernatant of rat liver homogenate led to the accumulation of [2-³H]-

Table 2. Formation of [2-³H]cholesta-7,9-dienol (VI) from 4,4'-dimethyl[2-³H]cholesta-7,9-dienol (II)

Aerobic incubations of 4,4'-dimethyl[2-³H]cholesta-7,9-dienol (II) (40 µg; 2 × 10⁶ d.p.m.) were carried out with a 10000g_{av.} supernatant of rat liver homogenate (5 ml). The sterols were extracted, purified by t.l.c., acetylated and separated by t.l.c. on silica gel/10% (w/w) AgNO₃. The fraction corresponding to cholesta-7,9-dienyl acetate was recrystallized after addition of unlabelled cholesta-7,9-dienyl acetate (7mg).

Steryl acetate isolated from AgNO ₃ t.l.c.	10 ⁻² × Total radioactivity (d.p.m.)	% of total acetylated extract
Cholesta-8,14-dienyl acetate	472	0.7
Cholesta-7,9-dienyl acetate	422	6.0
Cholesteryl acetate	591	8.0

Steryl acetate	No. of recrystallization	10 ⁻² × Sp. radioactivity (d.p.m./mg)
Cholesta-7,9-dienyl acetate	1	327
	2	239
	3	187
	4	132
	5	127
	6	127

cholesta-7,9-dienol (VII) in up to 6% yield (see Table 2). By using a boiled 10000g_{av.} supernatant or by doing the incubation anaerobically, there was no utilization of 4,4'-dimethylcholesta-7,9-dienol (II). Recrystallization of the presumptive cholesta-7,9-dienyl acetate with non-radioactive carrier (7mg) was carried out from cold methanol/diethyl ether. After an initial loss 40% of the radioactivity was retained to constant specific radioactivity. Mass-spectral analysis of the biosynthetic cholesta-7,9-dienyl acetate band indicated three compounds. The major component corresponded to authentic chemically synthesized cholesta-7,9-dienyl acetate and had a similar fragmentation pattern. A second component had the same mol.wt. as 4-monomethylcholesta-7,9-dienyl acetate. The other minor component was not investigated further. Subsequent further purification of the isolated sterol fraction gave a radioactive sample that was identical with pure cholesta-7,9-dienyl acetate when analysed by g.l.c.-mass spectrometry.

Loss of the C-4-methyl groups from 4,4'-dimethylcholesta-7,9-dienol (II) is probably catalysed by enzymes of the normal cholesterol-biosynthetic pathway which have quite a broad specificity (Miller & Gaylor, 1970; Gibbons, 1974). The C-4-methyl groups are removed sequentially, with the 4α-methyl groups lost first as CO₂. The resulting 4-monomethylcholesta-7,9-dienol then undergoes further demethylation to give cholesta-7,9-dienol (VI).

Conversion of various sterol substrates into cholesterol

The conversion of various sterols into cholesterol was studied in a 10000g_{av.} supernatant of rat liver homogenate. Dihydroagosterol (I) and 4,4'-dimethylcholesta-7,9-dienol (II) gave 11 and 14% conversions into cholesterol respectively, and this compares favourably with the 17 and 36% conversions of two well-established cholesterol precursors, 4,4'-dimethylcholesta-8,14-dienol and dihydrolanosterol, into cholesterol.

Discussion

The possible role of agosterol and dihydroagosterol (I) in cholesterol (VIII) biosynthesis is an intriguing one because of their minor, but not insignificant, occurrence with lanosterol and dihydrolanosterol in wool fat from sheep skin (Ruzicka *et al.*, 1945). This role is further emphasized by the apparent conversion of acetate into agosterol (Clayton & Bloch, 1956) and the conversion of dihydroagosterol (I) into cholesterol (VIII) in rat liver (Rahimtula *et al.*, 1969).

We have established that dihydroagosterol (I) is converted into cholesterol (VIII) via 4,4'-dimethylcholesta-7,9-dienol (II). This 7,9-diene accumulated in up to 27% yield during aerobic incubations. However, under anaerobic conditions the dihydroagosterol was not metabolized and was recovered unchanged. Coupled with the fact that 4,4'-dimethyl[2-³H]cholesta-7,9-dienol (II) is efficiently converted into cholesterol (VIII) in 14% yield, the above results provide strong evidence that this 7,9-diene is an intermediate in the biosynthesis of cholesterol (VIII) from dihydroagosterol (I).

The further metabolism of 4,4'-dimethylcholesta-7,9-dienol (II) is via cholesta-7,9-dienol (VI) and we have tentatively identified a monomethyl diene as a probable intermediate. The conversion of cholest-7,9-dienol (VI) into cholesterol (VIII) has already been shown to involve an initial isomerization to cholesta-8,14-dienol (VII) (Akhtar *et al.*, 1972).

Two points must be highlighted as a result of the above metabolic sequence. First, if the mechanism of the C-14-demethylation of dihydroagosterol (I) is identical with that of lanosterol then a Δ^{7,9,14}-triene intermediate must result, and this is the subject of the following paper (Tavarez *et al.*, 1977). Secondly, there is an apparent high degree of specificity of the enzyme system that isomerizes the Δ^{7,9}-sterol to the Δ^{8,14} product in that this system is completely specific for the non-dimethyl substrate. Such an absolute specificity is not seen for the isomerase that converts Δ⁸-sterols into the Δ⁷ series which is able to accept both 4,4'-dimethyl and non-dimethyl sterols as substrate. A similar specificity has been shown in the case of the metabolism of 4,4'-dimethylcholest-8(14)-

enol to cholesterol, where demethylation occurs before the metabolism of the $\Delta^{8(14)}$ double bond (Gibbons, 1974). However, because in this case both the demethylation and the metabolism of the double bond are aerobic processes one can only argue that demethylation is the kinetically more favoured event. On the other hand, the $\Delta^{7,9}$ - $\Delta^{8,14}$ isomerase action, which is an anaerobic reaction, does not occur with 4,4'-dimethylcholesta-7,9-dienol (II) as substrate under anaerobic conditions when the demethylation is prevented.

It is apparent from the above discussions that it is the $\Delta^{7,9}$ - $\Delta^{8,14}$ isomerase that is the one enzyme that is most likely to be unique to the proposed pathway (Scheme 1), whereas it may be argued that the other steps can be accommodated by existing enzymes since these are known to have relatively broad specificities. Possible mechanisms for this microsomal isomerase enzyme have been discussed (Rahimtula *et al.*, 1969) and are now under active investigation.

The precise role of agnosterol, especially in the skin, has yet to be determined, nor is there any information on its metabolism in this tissue. It may be relevant that in skin squalene cyclization results in the formation of the Δ^7 isomer of lanosterol (Gaylor, 1963). If this compound is formed directly as a result of squalene cyclization by the loss of a proton from C-7, then the C-9 hydrogen is left in the unnatural 9β -configuration. One possible way of rectifying this problem would be a subsequent dehydrogenation to give agnosterol.

Δ^9 -Lanosterol has also been identified in human meconium (Eneroth *et al.*, 1969), and could be derived directly from the cyclization of squalene as a result of proton loss from the C-11 position. Again dehydrogenation to give agnosterol is one route by which this compound could be further metabolized.

Finally, it is noteworthy that cholesta-8,14-dienol (VII) has been isolated as a significant component from Chang liver cells, an established cell line of human cells (Wilton, 1971). Although it can be argued that this sterol can be derived from the normal sterol intermediate, 4,4'-dimethylcholesta-8,14-dienol, by C-4-demethylation, it is surprising that the preferred pathway, namely reduction of the Δ^{14} -double bond, has not occurred before demethylation. However,

should $\Delta^{7,9}$ -sterols such as agnosterol have a significant role in cholesterol biosynthesis in these cells, then the pathway described above could explain the presence of cholesta-8,14-dienol (VII).

We thank Professor M. Akhtar for many helpful discussions and Dr. D. L. Corina for the mass-spectral measurements.

References

- Akhtar, M., Freeman, C. W., Rahimtula, A. D. & Wilton, D. C. (1972) *Biochem. J.* **129**, 225-229
- Alexander, K. A. (1974) Ph.D. Thesis, University of Southampton
- Birchenough, M. J. & McGhie, J. F. (1949) *J. Chem. Soc.* 2038-2039
- Bloxham, D. P., Wilton, D. C. & Akhtar, M. (1971) *Biochem. J.* **125**, 625-634
- Bowden, K., Heilbron, I. M., Jones, E. R. H. & Weedon, B. C. L. (1946) *J. Chem. Soc.* 39
- Clayton, R. B. & Bloch, K. (1956) *J. Biol. Chem.* **218**, 305-318
- Dewhurst, S. & Akhtar, M. (1967) *Biochem. J.* **105**, 1187-1194
- Eneroth, P., Gustafsson, J.-Å. & Nyström, E. (1969) *Eur. J. Biochem.* **11**, 456-464
- Gaylor, J. L. (1963) *J. Biol. Chem.* **238**, 1643-1655
- Gautschi, F. & Bloch, K. (1958) *J. Biol. Chem.* **233**, 1343-1347
- Gibbons, G. F. (1974) *Biochem. J.* **144**, 59-68
- Heusser, H., Heusler, K., Eichenbergen, K., Honneger, C. G. & Jager, O. (1952) *Helv. Chim. Acta* **35**, 295
- Miller, W. L. & Gaylor, J. L. (1970) *J. Biol. Chem.* **245**, 5369-5374
- Miller, W. L., Kalafer, M. E., Gaylor, J. L. & Delwiche, C. V. (1967) *Biochemistry* **6**, 2673-2678
- Rahimtula, A. D., Wilton, D. C. & Akhtar, M. (1969) *Biochem. J.* **112**, 545-546
- Ruzicka, L., May, E. & Muhr, A. C. (1945) *Helv. Chim. Acta* **27**, 472-479
- Tavares, I. A. F., Munday, K. A. & Wilton, D. C. (1977) *Biochem. J.* **166**, 17-20
- Watkinson, I. A., Wilton, D. C., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **121**, 131-137
- Wilton, D. C. (1971) *Biochem. J.* **125**, 1153-1156
- Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968) *Biochem. J.* **106**, 803-810