The Metabolic Sequence by which some 4,4-Dimethyl Sterols are Converted into Cholesterol

By GEOFFREY F. GIBBONS

Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital,
London W12 0HS, U.K.

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Cholest-8(14)-enol is the major radioactive component of the 4-di-demethyl sterol fraction biosynthesized from 4,4-dimethyl[2-³H₂]cholest-8(14)-enol by rat liver microsomal fractions, and therefore the first steps in the biosynthesis of cholesterol from the latter compound probably involve removal of the 4-methyl groups. 4,4-Dimethylcholesta-8,14-dienol therefore is not an intermediate in this process, although its presence in the incubation medium at a concentration of 0.146mM almost completely inhibits the demethylation of 4,4-dimethyl[2-³H₂]cholest-8(14)-enol. Nor is cholesta-8,14-dienol an intermediate in the conversion of cholest-8(14)-enol into cholest-7-enol and cholesterol. With 4,4-dimethyl[2-³H₂]cholesta-8,14-dienol as the cholesterol precursor, 4,4-dimethylcholest-8(9)-enol becomes heavily labelled and there is very little radioactivity associated with cholesta-8,14-dienol. In this case, the most heavily labelled 4-di-demethyl sterols are cholest-7-enol and cholesterol with the former predominating. There is little or no radioactivity associated with cholest-8(14)-enol. A similar labelling pattern amongst the 4-di-demethyl sterols was observed with dihydro[¹⁴C]lanosterol as the precursor. The first step therefore in the synthesis of cholesterol from the 4,4-dimethyl[2-³H₂]dienol is reduction of the $\Delta^{14(15)}$ bond and not removal of the 4 α -methyl group. Depending on the nature of the precursor, addition of the soluble fraction of the cell to the microsomal fraction resulted in a two- to four-fold stimulation of 4-di-demethyl sterol biosynthesis from the 4,4-dimethyl sterols studied. Under these conditions, 4,4-dimethylcholesta-8,14-dienol is the most efficient precursor of cholesterol and cholest-7-enol, and dihydrolanosterol is better than 4,4-dimethylcholest-8(14)-enol.

The mechanism and sequence of events by which the 14 α -methyl group of lanosterol (compound Ia, Fig. 1) is removed are not completely established despite many recent investigations. It seems likely that the methyl carbon is eliminated as formic acid (Alexander *et al.*, 1972) concomitant with the ultimate formation of a $\Delta^{8,14}$ diene or $\Delta^{8,14,24}$ triene (compound IIa or IIc, Fig. 1). The intermediacy of a $\Delta^{8(14)}$ sterol of the type shown in Fig. 1 (structure III) in this process is a matter of controversy. Sterols of this type occur naturally (Lutsky & Schroepfer, 1969; Zalkow *et al.*, 1968; Fioriti *et al.*, 1970) and both structures IIIa (Fried *et al.*, 1968; Fiecchi *et al.*, 1970; Paoletti *et al.*, 1971) and IIIb (Lee *et al.*, 1969b; Lee & Schroepfer, 1968) are converted into cholesterol by cell-free systems. The reported formation of radioactive compound IIb during cholesterol biosynthesis from labelled compound IIIb (Lutsky & Schroepfer, 1971) suggests the possibility of a biosynthetic sequence involving these compounds, at least at the 4-di-demethyl sterol level. However, at the 4,4-dimethyl sterol level, the stage at which the monoene-diene transformation should occur physiologically, although exogenously added non-radio-

active 4,4-dimethyldiene (compound IIa) 'traps' radioactivity from labelled dihydrolanosterol (compound Ib) (Fiecchi *et al.*, 1969; Watkinson & Akhtar, 1969; Watkinson *et al.*, 1971; Alexander *et al.*, 1971), the non-radioactive 4,4-dimethylmonoene (compound IIIa) does not (Alexander *et al.*, 1971; Fiecchi *et al.*, 1972). Nor does the addition of non-radioactive diene IIa result in an accumulation of radioactivity from the labelled monoene IIIa (Fiecchi *et al.*, 1970; Paoletti *et al.*, 1971), although these authors observed a large decrease in the formation of labelled cholesterol under these conditions. This observation, in itself, does not completely rule out the possibility that $\Delta^{8(14)}$ sterols participate in cholesterol biosynthesis and requires further explanation. Also, the convertibility of $\Delta^{8(14)}$ sterols into $\Delta^{8,14}$ sterols at the 4-di-demethyl level is difficult to reconcile with the above experiments at the 4,4-dimethyl sterol level. In an attempt to clarify this situation, and to assess the relative importance of structures IIa and IIIa in the major pathway of cholesterol biosynthesis, the rates of methyl group loss and double-bond rearrangement, and the sequence with which these events occur during cholesterol synthesis from these

Fig. 1. Structures of some known and proposed 4,4-dimethyl sterol precursors of cholesterol

compounds, have been studied *in vitro*. This has been compared with the rate and postulated sequence by which more established intermediates such as lanosterol (Ia) and dihydrolanosterol (Ib) are converted into cholesterol.

Experimental

Methods

The preparation of cell-free systems, incubation procedures, isolation of the total lipid fraction, separation of 4,4-dimethyl sterols (lanosterol and companion C_{30} and C_{29} sterols) from 4-di-demethyl sterols (cholesterol and companion C_{27} sterols) and steroidal 3-ketones (t.l.c. on silica gel H in chloroform as the mobile phase, system 1), emulsification of sterol substrates, g.l.c., acetylation procedure and measurement of radioactivity have been described previously (Gibbons & Mitropoulos, 1973*a,b*). The only deviation from these procedures is that incubations were conducted in flasks covered with Parafilm (Gallenkamp, London E.C.2, U.K.), with air as the

gas phase. Unless otherwise stated, the incubation medium contained 1.2 ml of microsomal fraction, and, where appropriate, 3.0 ml of S_{104} (the supernatant fraction obtained after spinning the S_{10} fraction for 1.0 h at $105000g_{av.}$) in a total volume of 5.0 ml. In each experiment, a zero-time control was carried out. This flask contained all the components of the incubated samples, but enzymic activity was destroyed immediately after addition of the substrate, by addition of a mixture of chloroform and methanol (2:1, v/v). Immediately after incubation, appropriate carrier sterols were added to the mixture.

The components of the 4-di-demethyl sterol fraction were separated by acetylation and by chromatography on thin-layer plates of AgNO_3 -impregnated alumina (Gibbons *et al.*, 1973), with a mixture of toluene and hexane (1:3, v/v) as the moving phase (system 3). The steryl acetates were made visible by spraying with a solution of Rhodamine 6G in acetone (0.1%, w/v). In this system, the R_f values (cholesteryl acetate = 1) of cholest-7-enyl acetate, cholest-8(14)-enyl acetate and cholesta-8,14-dienyl acetate were 1.27, 1.53 and 0.23 respectively. In some cases, further

purification of the cholesta-8,14-dienyl acetate fraction was achieved by chromatography on AgNO₃-impregnated plates of silica gel H or alumina, with a mixture of toluene and hexane (1:1, v/v) as the moving phase (systems 2 and 4 respectively). Fractions containing 4,4-dimethylcholesta-8,14-dienol were isolated from the 4,4-dimethyl sterol fraction obtained after chromatography in system 1, by acetylation and further chromatography in system 2. After each chromatographic step in the purification procedures, portions of each fraction were removed for radioassay before further chromatography was undertaken.

Protein was measured by the method of Cleland & Slater (1953).

Chemical syntheses

Non-radioactive 4,4-dimethylcholesta-5,7-dien-3-one, 4,4-dimethyl-5 α -cholesta-8,14-dien-3 β -ol, 4,4-dimethyl-5 α -cholest-8(14)-en-3 β -ol and 4,4-dimethyl-5 α -cholest-8(9)-en-3 β -ol were prepared as described previously (Gibbons & Mitropoulos, 1973a).

5 α -Cholesta-8,14-dien-3 β -ol. This was prepared by HCl isomerization of cholesta-5,7-dien-3 β -ol (4.0g) by the method of Fieser & Ourisson (1953). Extraction followed by a threefold crystallization from methanol, gave 5 α -cholesta-8,14-dien-3 β -ol (0.32g), $\lambda_{\max.}$, 250nm; ϵ_{250} , 17100 litre·mol⁻¹·cm⁻¹; m.p., 114–115°C (m.p. 3-acetate, 97–99°C). The reported physical constants for this material are: m.p., 116–117°C (m.p. 3-acetate, 99–100°C); $\lambda_{\max.}$, 250nm; ϵ_{250} , 18100 litre·mol⁻¹·cm⁻¹ (Fieser & Ourisson, 1953).

5 α -Cholest-8(14)-en-3 β -ol. Hydrogenation of a solution of cholesta-5,7-dienyl acetate (400mg) in acetic acid (75ml) in the presence of Adams catalyst (100mg) for 3h at 118°C gave a material which had no u.v. absorption at 283nm. Recrystallization of the product from a mixture of chloroform and methanol gave 5 α -cholest-8(14)-enyl acetate (200mg), m.p., 74–76°C [reported, 77.5–78.5°C (Lee *et al.*, 1969a)]. G.l.c. on SE-30 showed the presence of only one peak with a relative retention time (cholesterol = 1) of 1.436. Alkaline hydrolysis of this material and two crystallizations from methanol gave 5 α -cholest-8(14)-en-3 β -ol, m.p., 118–120°C [reported, 120–121°C (Lee *et al.*, 1969a)].

4,4-Dimethyl-5 α -[2-³H₂]cholest-8(14)-en-3 β -ol. 4,4-Dimethyl-5 α -cholest-8(14)-en-3 β -ol (51mg, m.p. 143–144°C) was oxidized to the 3-ketone by the method of Djerassi *et al.* (1956). The product showed only one band on t.l.c. (system 1) with an R_F value of 0.67 (R_F lanosterol, 0.42). Crystallization from methanol gave plates (31mg), m.p. 94–96°C [reported, 98–99°C (Fried & Brown, 1967)]. I.r. analysis showed a strong band at 1706cm⁻¹ and a complete absence of absorption in the region 4000–3000cm⁻¹. A portion of this

material (7.5mg) was dissolved in hexane (1.0ml) and applied to a column of basic alumina (3.0g, 160mm×7mm) which had been deactivated with tritiated water (0.2ml, 2.5Ci/ml) and which had been allowed to equilibrate for 20h before the passage of 30ml of hexane (Klein & Knight, 1965). After application of the 3-ketone, the column was eluted with hexane (60ml), a mixture of ether and hexane (1.5:98.5, v/v; 20ml), and finally, a further mixture of ether and hexane (3:97, v/v; 20ml). Fractions of 2.0ml were taken during the chromatography and a portion of each fraction was taken for radioassay. The 4,4-dimethyl[2-³H₂]enone was eluted in the fractions containing ether-hexane (1.5:98.5, v/v). These fractions were combined, and the solvent was evaporated to give a total radioactivity of 5.101×10⁸d.p.m. To this material (7.8mg) was added non-radioactive 4,4-dimethyl-5 α -cholest-8(14)-en-3-one (5.2mg); it was then reduced with a suspension of lithium aluminium hydride (60mg) in dry ether (7.0ml). After extraction and t.l.c. (system 1) only one band appeared with the same R_F value as lanosterol. This contained 11.8mg (3.568×10⁸d.p.m.). After crystallization from methanol, the material weighed 8.51mg (2.997×10⁸d.p.m.). The compound was further purified by acetylation and t.l.c. in system 2. Only one band appeared which, after elution, hydrolysis, t.l.c. in system 1 and finally, crystallization from methanol yielded 3.37mg (1.307×10⁸d.p.m.), m.p., 143–144°C [reported, 142–143°C (Gautschi & Bloch, 1958)].

4,4-Dimethyl-5 α -[2-³H₂]cholesta-8,14-dien-3 β -ol. 4,4-Dimethylcholesta-5,7-dien-3-one (7.5mg) was tritiated in the same way as described above. The [2-³H₂]dienone, eluted in the fractions containing ether-hexane (1.5:98.5, v/v), weighed 7.4mg (4.033×10⁸d.p.m.). This was diluted with non-radioactive material (5.0mg) and reduced with lithium aluminium hydride as described above. The extracted product (3.355×10⁸d.p.m.) was chromatographed in system 1 and the band with the same R_F value as lanosterol was eluted (2.959×10⁸d.p.m.). HCl isomerization of the [2-³H₂]dienol (Fieser & Ourisson, 1953) gave, after extraction, t.l.c. (system 1) and recrystallization of the eluted material from methanol, a compound (5.6mg, 1.832×10⁸d.p.m.), with $\lambda_{\max.}$, 250nm; ϵ_{250} , 16400 litre·mol⁻¹·cm⁻¹; m.p., 139–141°C. Further purification was effected by acetylation and t.l.c. (system 2). Only one u.v.-absorbing band was observed. The material was eluted, hydrolysed and rechromatographed in system 1. Elution of the band with the same R_F value as lanosterol, and recrystallization from methanol, gave pure 4,4-dimethyl-5 α -[2-³H₂]cholesta-8,14-dien-3 β -ol (2.42mg, 8.11×10⁷d.p.m.).

Materials

The sources of materials used in this work, and of the preparation of dihydro[1,7,15,22,26,30-¹⁴C₆]-

Table 1. *Distribution of radioactivity amongst the microsomal metabolites of 4,4-dimethyl[2-³H₂]cholest-8(14)-en-3 β -ol, 4,4-dimethyl[2-³H₂]cholesta-8,14-dien-3 β -ol and dihydro[¹⁴C]lanosterol*

Incubations were done at 37°C for 40 min in the presence of microsomal fraction (1.2 ml), 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-MgCl₂, 1 unit of glucose 6-phosphate dehydrogenase (1 μ mol of NADPH generated/min) and 0.1 M-potassium phosphate buffer (pH 7.4) in a total volume of 5.0 ml. The incubations were started by addition of the substrate emulsion in potassium phosphate buffer, pH 7.4 (0.1 ml). Immediately after incubation, carrier sterols were added as follows: cholest-7-en-3 β -ol and cholest-8(14)-en-3 β -ol (0.5 mg of each to all flasks); 4,4-dimethylcholest-8(14)-en-3 β -ol (2.0 mg to flasks 37a and 41a); 4,4-dimethylcholesta-8,14-dien-3 β -ol (2.0 mg to flasks 37b and 41b) and dihydrolanosterol (1.0 mg to flask 47). The total lipid fraction was isolated by extracting with a mixture of chloroform and methanol (2:1, v/v) and the 4-di-demethyl sterols were isolated from the other steroids by t.l.c. in system 1. The components of the 4-di-demethyl sterol fraction were isolated by acetylation followed by t.l.c. in system 3. Each incubation was conducted in duplicate and the reported values are an average of the two.

Expt. no.	Precursor and amount incubated (d.p.m., nmol)	Percentage of incubated radioactivity associated with:				
		4,4-Dimethyl sterols	4-Di-demethyl sterols	Cholest-8(14)-en-3 β -ol	Cholest-7-en-3 β -ol	Cholesterol
37a	4,4-Dimethyl[2- ³ H ₂]cholest-8(14)-en-3 β -ol, 6.46 $\times$ 10 ⁵ , 40.2	67.0	5.1	2.2	0.8	0.3
37b	4,4-Dimethyl[2- ³ H ₂]cholesta-8,14-dien-3 β -ol, 6.42 $\times$ 10 ⁵ , 46.4	51.9	20.7	0.4	10.2	3.1
41a	4,4-Dimethyl[2- ³ H ₂]cholest-8(14)-en-3 β -ol, 1.41 $\times$ 10 ⁶ , 87.8	74.7	3.3	0.9	0.7	0.3
41b	4,4-Dimethyl[2- ³ H ₂]cholesta-8,14-dien-3 β -ol, 1.31 $\times$ 10 ⁶ , 94.7	49.2	15.7	0.2	9.1	1.7
47*	Dihydro[¹⁴ C]lanosterol, 2.00 $\times$ 10 ⁵ , 77.1	80.4	9.1	0.4	5.4	2.1

* Incubated for 1 h. Single incubation only. Total volume = 5.2 ml.

Table 2. *Recrystallization to constant specific radioactivity of the acetates of the 4-di-demethyl sterols biosynthesized from labelled 4,4-dimethyl sterols*

The radioactive cholest-8(14)-enyl acetate, cholest-7-enyl acetate and cholesteryl acetate fractions labelled after incubation with the radioactive 4,4-dimethyl sterols were isolated by t.l.c. in system 3. To each fraction was added the appropriate non-radioactive steryl acetate and each mixture was recrystallized several times. After each crystallization, the weight of the crystals was determined and a portion of each was removed for measurement of radioactivity.

4-Di-demethyl sterol product	No. of recrystallizations	Labelled from 4,4-dimethyl[2- ³ H ₂]cholest-8(14)-en-3 β -ol, specific radioactivity (d.p.m./mg)	Labelled from 4,4-dimethyl[2- ³ H ₂]cholesta-8,14-dien-3 β -ol, specific radioactivity (d.p.m./mg)	Labelled from dihydro[¹⁴ C]lanosterol, specific radioactivity (d.p.m./mg)
Cholest-8(14)-enyl acetate	0	1056.7	—	—
	1	1022.8	—	—
	2	943.2	—	—
	3	900.7	—	—
	4	925.4	—	—
Cholest-7-enyl acetate	0	277.4	2442.1	221.2
	1	253.5	1953.6	176.5
	2	227.2	1789.9	158.2
	3	244.8	1880.9	156.2
	4	243.3	1800.1	—
Cholesteryl acetate	0	63.9	441.3	86.1
	1	49.6	395.8	67.9
	2	56.0	384.8	75.7
	3	56.7	411.9	85.4
	4	50.3	378.4	—

lanosterol (referred to as dihydro[^{14}C]lanosterol) have been reported elsewhere (Gibbons & Mitropoulos, 1973a,b). The latter material was diluted with the required amount of non-radioactive dihydro-lanosterol before incubation. In addition, tritiated water (5.0Ci/ml) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., cholest-7-enol (m.p., 120–122°C) was purchased from Steroids Ltd. (Croydon, Surrey, U.K.) and basic alumina was obtained from M. Woelm, Eschwege, Germany. Toluene was of AnalaR grade and was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Results

Metabolism of 4,4-dimethyl sterols by rat liver microsomal fraction

Microsomal fractions of rat liver were incubated independently with dihydro[^{14}C]lanosterol, 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-enol and 4,4-dimethyl[2- $^3\text{H}_2$]cholesta-8,14-dienol. Incubations with the ^3H -labelled substrates were carried out at two different substrate concentrations. After the addition of carrier sterols (Table 1), the fractions containing the 4,4-dimethyl sterols, steroidal-3-ketones and 4-di-demethyl sterols were separated from the isolated total lipid material by t.l.c. in system 1, and a portion of each was removed for measurement of radioactivity. The radioactive components of each 4-di-demethyl sterol fraction were separated by t.l.c. in system 3 after acetylation, and the distribution of radioactivity amongst these constituent sterols is shown in Table 1. The major radioactive 4-di-demethyl sterol product of 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-enol metabolism had the chromatographic properties of cholest-8(14)-enol (isolated as the 3 β -acetate derivative). Cholesterol and cholest-7-enol (isolated as the 3 β -acetate derivatives) were labelled to a much smaller extent. The radiochemical identity of each of these compounds was confirmed by addition of the appropriate carrier material to each radioactive fraction, followed by measurement of specific radioactivity after successive recrystallizations (Table 2). In a similar experiment, after acetylation and t.l.c. (system 2) of the 4,4-dimethyl sterol fraction, there were 8800 d.p.m. (1.51% of the incubated radioactivity) associated with the 4,4-dimethylcholesta-8,14-dienyl acetate band. This was diluted with non-radioactive material to give an initial specific radioactivity of 367 d.p.m./mg. After five recrystallizations, the specific radioactivity decreased progressively (126, 70, 55, 43 and 37 d.p.m./mg respectively).

Cholest-7-enol and cholesterol (isolated as the 3-acetate derivatives) were the major radioactive 4-di-demethyl sterols after incubation with either dihydro[^{14}C]lanosterol or 4,4-dimethyl[2- $^3\text{H}_2$]chole-

sta-8,14-dienol (Table 1). However, cholest-7-enol was more highly labelled than cholesterol in each case. Addition of the appropriate non-radioactive steryl acetate to each radioactive fraction, obtained after chromatography of the 4-di-demethyl steryl acetate mixture in system 3, followed by recrystallization and measurement of specific radioactivity, showed that most of the radioactivity remained associated with these compounds (Table 2). In both types of incubation, less than 0.4% of the incubated radioactivity had the chromatographic mobility of $\Delta^{8(14)}$ sterols, and this was probably due to contamination by the highly labelled cholest-7-enyl acetate fraction.

In a similar experiment, 4,4-dimethyl[2- $^3\text{H}_2$]cholesta-8,14-dienol (1.343×10^6 d.p.m.; 97.1 nmol) was incubated with microsomal fraction (1.2 ml) for 40 min. The incubation was carried out in duplicate, and at the end of the incubation period 4,4-dimethylcholesta-8,14-dienol (2.0 mg), 4,4-dimethylcholest-8(9)-enol (1.0 mg), cholesta-8,14-dienol (2.0 mg), cholesta-5,7-dienol (3.0 mg), cholest-7-enol and cholest-8(14)-enol (0.5 mg of each) were added and the radioactive 4,4-dimethyl and 4-di-demethyl sterols were isolated. After chromatography of the acetylated 4-di-demethyl sterol fraction (76350 d.p.m. av.) in system 3, the dienic steryl acetates (29200 d.p.m. av.) were rechromatographed in system 4. The isolated cholesta-8,14-dienyl acetate contained 16800 d.p.m. (av.). To this material was added non-radioactive cholesta-8,14-dienyl acetate to give an initial specific radioactivity of 593 d.p.m./mg, and the mixture was recrystallized four times. After an initial decrease, the specific radioactivity remained more or less constant after successive recrystallizations (255, 211, 220 and 214 d.p.m./mg respectively). On the basis of the value for the final constant specific radioactivity therefore only 0.45% of the incubated radioactivity was associated with cholesta-8,14-dienol. In contrast, chromatography of the acetylated 4,4-dimethyl sterol fraction (1.00×10^6 d.p.m. av.) in system 2 showed that 182300 d.p.m. av. (13.57% of the incubated radioactivity) was associated with 4,4-dimethylcholest-8(9)-enyl acetate.

4,4-Dimethyl sterol metabolism by microsomal preparations in the presence of S_{104} fraction

To determine whether the distribution of radioactivity amongst the 4-di-demethyl sterol products of 4,4-dimethyl sterol metabolism was influenced by the presence of non-catalytic soluble carrier protein (Ritter & Dempsey, 1971; Scallen *et al.*, 1971), incubations were carried out with each of the labelled substrates in the presence and absence of the S_{104} fraction. The results of these experiments are presented in Table 3, which shows that the rate of synthesis of the 4-di-demethyl sterols from all substrates is considerably increased in the presence of the S_{104}

Table 3. *Effect of the soluble fraction of the cell (S₁₀₄) on the microsomal metabolism of 4,4-dimethyl sterols*

Incubations were done for 1 h at 37°C in the presence of microsomal fraction (1.2 ml) or microsomal fraction (1.2 ml) plus S₁₀₄ fraction (3.0 ml) in a total volume of 5.2 ml. The concentrations of cofactors were the same as those given in Table 1. Each substrate was suspended with the aid of Tween 80 in potassium phosphate buffer (pH 7.4). The concentration of Tween 80 was 3.0 mg/flask. The microsomal protein concentration was 10.50 mg/ml (Expt. 44) and 22.84 mg/ml (Expt. 47). The S₁₀₄ fraction contained 12.44 mg (Expt. 44) and 13.24 mg (Expt. 47) of protein/ml. Immediately after incubation, carrier sterols were added to the incubation contents as follows: cholest-7-en-3 β -ol and cholest-8(14)-en-3 β -ol (0.5 mg of each to all flasks); 4,4-dimethylcholest-8(14)-en-3 β -ol (2.0 mg to flasks 44a, 44b and 47a); 4,4-dimethylcholesta-8,14-dien-3 β -ol (2.0 mg to flasks 44a, 44b, 47b, 47c); cholesta-8,14-dien-3 β -ol (2.0 mg to flasks 44a and 47a); and dihydrolanosterol (1.0 mg to flasks 47d and 47e). The radioactive sterol fractions were isolated as described in Table 1.

Expt. no.	Precursor and amount incubated (d.p.m., nmol)	Enzyme system	Percentage of incubated radioactivity associated with:					
			4,4-Dimethyl sterols	4-Di-demethyl sterols	Cholest-8(14)-en-3 β -ol	Cholest-7-en-3 β -ol	Cholesterol	
44a	* 4,4-Dimethyl[2- ³ H ₂]cholest-8(14)-en-3 β -ol, 6.00 $\times$ 10 ⁵ , 37.4	Microsomal fraction	78.9	4.5	2.5	0.5	0.3	
44b	4,4-Dimethyl[2- ³ H ₂]cholest-8(14)-en-3 β -ol, 6.00 $\times$ 10 ⁵ , 37.4	Microsomal fraction + S ₁₀₄	67.9	9.6	4.3	2.3	1.1	
47a	4,4-Dimethyl[2- ³ H ₂]cholest-8(14)-en-3 β -ol, 1.276 $\times$ 10 ⁶ , 79.5	Microsomal fraction + S ₁₀₄	60.3	17.6	4.9	6.2	3.9	
47b	4,4-Dimethyl[2- ³ H ₂]cholesta-8,14-dien-3 β -ol, 1.111 $\times$ 10 ⁶ , 80.1	Microsomal fraction	53.5	24.7	0.3	16.2	4.1	
47c	4,4-Dimethyl[2- ³ H ₂]cholesta-8,14-dien-3 β -ol, 1.111 $\times$ 10 ⁶ , 80.1	Microsomal fraction + S ₁₀₄	23.8	51.8	1.2	27.9	19.0	
47d	Dihydro[¹⁴ C]lanosterol, 2.00 $\times$ 10 ⁵ , 77.1	Microsomal fraction	80.4	9.2	0.4	5.4	2.1	
47e	Dihydro[¹⁴ C]lanosterol, 2.00 $\times$ 10 ⁵ , 77.1	Microsomal fraction + S ₁₀₄	46.8	36.6	0.8	17.6	14.6	

* Total volume, 5.0 ml.

Table 4. *Effect of non-radioactive cholesta-8,14-dien-3 β -ol and 4,4-dimethylcholesta-8,14-dien-3 β -ol on the microsomal metabolism of 4,4-dimethyl[2-³H₂]cholest-8(14)-en-3 β -ol*

Microsomal fraction (1.2 ml) in a total volume of 5.0 ml) was incubated for 1 h at 37°C with 4,4-dimethyl[2-³H₂]cholest-8(14)-en-3 β -ol (6.00 $\times$ 10⁵ d.p.m., 37.4 nmol), either alone or in the presence of 4,4-dimethylcholesta-8,14-dien-3 β -ol or cholesta-8,14-dien-3 β -ol. Where applicable, the radioactive and non-radioactive materials were emulsified together before addition to the enzyme system. The concentration of Tween 80 was 3.0 mg/flask. The microsomal protein concentration was 10.50 mg/ml. Each incubation was carried out in duplicate, and the reported values represent an average of the two values.

Non-radioactive material added (μ mol)	Percentage of incubated radioactivity associated with:					
	Cholest-8(14)-en-3 β -ol	Cholest-7-en-3 β -ol	Cholesterol	Cholesta-8,14-dien-3 β -ol	4,4-Dimethylcholesta-8,14-dien-3 β -ol	4,4-Dimethylcholest-8(14)-en-3 β -ol
—	2.5	0.5	0.3	$\leq$ 0.1	2.3	71.0
4,4-Dimethylcholesta-8,14-dien-3 β -ol, 0.73	0.4	<0.1	0.1	No detectable radioactivity	0.9	88.7
Cholesta-8,14-dien-3 β -ol, 0.78	1.3	0.2	0.1	No detectable radioactivity	1.2	79.8

fraction; from dihydro[^{14}C]lanosterol, a fourfold stimulation was observed; from 4,4-dimethyl[2- $^3\text{H}_2$]-cholesta-8,14-dienol and 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-enol, there was a 2.10- and 2.15-fold increase respectively. Regardless of the nature of the substrate, the amount of labelled cholesterol relative to labelled cholest-7-enol was greater in the presence of the S_{104} fraction. When 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-enol was the substrate, although the sum of the radioactivities of cholesterol and cholest-7-enol increased relative to that of cholest-8(14)-enol in the presence of S_{104} , the latter sterol still contributed a large proportion to the total radioactivity of the 4-di-demethyl sterol fraction. 4,4-Dimethyl[2- $^3\text{H}_2$]cholesta-8,14-dienol in the presence of the microsomal preparation plus S_{104} was converted into 4-di-demethyl sterols at a greater rate (51.8%) than was dihydro[^{14}C]lanosterol (36.6%), which itself was converted at a greater rate than was 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-enol (17.6%, Table 3).

Effect of non-radioactive 4,4-dimethylcholesta-8,14-dien-3 β -ol and cholesta-8,14-dien-3 β -ol on the rate of 4-di-demethyl sterol biosynthesis from 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-en-3 β -ol

Rat liver microsomal preparations were incubated in the presence of 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-en-3 β -ol either alone or in the presence of relatively large amounts of non-radioactive 4,4-dimethylcholesta-8,14-dien-3 β -ol or cholesta-8,14-dienol. The residual radioactive 4,4-dimethyl sterol fraction was separated from the fractions containing 4-di-demethyl sterols and steroidal 3-ketones by t.l.c. in system 1. The constituents of each of the first two fractions were separated after acetylation followed by chromatography in systems 2 and 3 respectively. The cholesta-8,14-dienyl acetate so obtained after chromatography in system 3 was further purified by two successive chromatographic purifications in system 2. The results of this experiment are expressed in Table 4. In the presence of non-radioactive 4,4-dimethylcholesta-8,14-dien-3 β -ol, the rate of synthesis of cholesterol and its 4-di-demethyl sterol precursors, cholest-7-enol and cholest-8(14)-en-3 β -ol, was substantially decreased and there was an increase in the radioactivity of the residual substrate. There was no increase in the amount of radioactivity associated with 4,4-dimethylcholesta-8,14-dien-3 β -ol. That the observed radioactivity of this fraction was not due to 4,4-dimethylcholesta-8,14-dien-3 β -ol has been demonstrated by the complete loss of radioactivity after addition of carrier material followed by several recrystallizations (see above). Addition of non-radioactive cholesta-8,14-dien-3 β -ol also inhibited the conversion of 4,4-dimethyl[2- $^3\text{H}_2$]cholest-8(14)-en-3 β -ol into the 4-di-demethyl sterols, but to a much smaller degree. There was no increase in the

amount of radioactivity associated with cholesta-8,14-dienol.

Discussion

The results show that, although the 4,4-dimethyl[2- $^3\text{H}_2$]monoene (structure IIIa) is a reasonably efficient precursor of 4-di-demethyl sterols, its convertibility into cholesterol (compound XV, Scheme 1) is poor. It seems likely that, as cholest-8(14)-en-3 β -ol (compound IIIb) accumulates during this process, this compound is an intermediate in the conversion of compound IIIa into cholest-7-en-3 β -ol (compound XIV, Scheme 1) and cholesterol, and that the rearrangement of the nuclear double bond from the $\Delta^{8(14)}$ position to, ultimately, the Δ^7 position, is rate-limiting. Hence the loss of the 4-methyl groups of structure IIIa precedes the rearrangement of the double bond, and the first reaction therefore in the metabolism of this compound is oxidation of the 4 α -methyl group (IIIa $\rightarrow$ V, Scheme 1). However, it has been suggested (Ritter *et al.*, 1972) that the metabolic sequence by which various precursors are converted into cholesterol may vary depending on the concentration of non-catalytic soluble carrier proteins (Ritter & Dempsey, 1971; Scallen *et al.*, 1971) in the incubation medium. Although in the present case, supplementation of the microsomal fraction with the soluble fraction of the cell (S_{104}) considerably increased the rate of cholesterol synthesis from the 4,4-dimethyl[2- $^3\text{H}_2$]monoene (structure IIIa), cholest-8(14)-enol remained a predominant constituent of the 4-di-demethyl sterol fraction, suggesting that the soluble carrier proteins had no effect on the sequence of events. Under these circumstances, however, the concentration of cholest-7-en-3 β -ol, a known precursor of cholesterol, diminished relative to that of cholesterol itself, and a similar increase in the cholesterol/cholest-8(14)-en-3 β -ol ratio provides further evidence for the existence of a product-precursor relationship between the two. If this is the case, then the proportion of radioactive cholesterol in the 4-di-demethyl sterol complex biosynthesized from the 4,4-dimethyl[2- $^3\text{H}_2$]monoene will depend on the activity of the enzymes and the length of incubation time. This may go some way towards explaining the apparent discrepancy between the work of Akhtar's group (Alexander *et al.*, 1971), who demonstrated that compound IIIa was a less efficient precursor of cholesterol than was dihydrolanosterol (Ib), an observation confirmed by the present work, and that of Fried *et al.* (1968), who observed the opposite. The results of the latter authors are, however, ambiguous in any case, owing to the fact that the concentration of the incubated compound Ib was very different from that of compound IIIa.

In the presence of non-radioactive 4,4-dimethyl-diene (IIa) the rate of biosynthesis of every 4-di-

demethyl sterol from radioactive compound IIIa was decreased to a very low value (Table 4), and compound IIa itself did not accumulate radioactivity. This confirms and extends previous observations in this respect (Paoletti *et al.*, 1971), but the present results demonstrate that, in view of the decrease in the concentration of the radioactive steroidal 3-ketone fraction (probably structure VIII, Scheme 1) and the increase in residual radioactive compound IIIa under these circumstances, non-radioactive compound IIa exerts its inhibitory effect at the 4,4-dimethyl sterol-4 α -demethylase stage, presumably by virtue of the 4,4-dimethyl grouping of compound IIa competing with that of compound IIIa for the demethylase enzymes. If cholest-8(14)-enol is an intermediate in the conversion of compound IIIa into cholesterol, it seemed possible that cholesta-8,14-dien-3 β -ol (IIb) may be involved in the rearrangement of the $\Delta^{8(14)}$ bond to the Δ^8 or Δ^7 positions (Lutsky & Schroepfer, 1971). However, addition of non-radioactive compound IIb to a microsomal system containing radioactive compound IIIa decreased the rate of 4-di-demethyl sterol synthesis, but the ratio of cholest-8(14)-enol to cholest-7-enol did not increase, as would be expected owing to isotope dilution, if cholesta-8,14-dien-3 β -ol was an intermediate between the two. Nor did the latter sterol accumulate any measurable activity under these conditions. The mechanism of conversion of compound IIIb to compound XIII and/or compound XIV (Scheme 1) therefore remains unknown. Cholesta-8,14-dien-3 β -ol is not involved, but an oxygenated intermediate, possibly cholest-8(14)-en-3 β ,15 ξ -diol (Y, Scheme 1 of Huntoon & Schroepfer, 1970) may participate in the process.

In view of the efficient conversion of cholesta-8,14-dien-3 β -ol (compound IIb) into cholesterol by liver enzymes (Lutsky & Schroepfer, 1968, 1970; Canonica *et al.*, 1968; Akhtar *et al.*, 1968; Watkinson *et al.*, 1971) and the formation of this compound during cholesterol biosynthesis from [2- 14 C]mevalonic acid by Chang liver cells (Wilton, 1971), it was of interest to determine whether this was an obligatory physiological precursor of cholesterol. However, the present results indicate that the first reaction in the metabolism of the 4,4-dimethyl[2- 3 H $_2$]diene (compound IIa) to cholesterol is reduction of the Δ^{14} bond to give 4,4-dimethylcholest-8(9)-enol (compound IV) and not removal of the methyl groups to give compound IIb (Scheme 1). This was evidenced by the relative amounts of these compounds isolated after metabolism of compound IIa in which 13.57 and 0.45% of the incubated radioactivity was associated with compounds IV and IIb respectively. This is consistent with the postulated sequence by which lanosterol is converted into cholesterol and in the present work the radioactivity distribution pattern amongst the 4-di-demethyl sterol products of

dihydro[14 C]lanosterol metabolism was very similar to that observed when the 4,4-dimethyl[2- 3 H $_2$]diene (structure IIa) was the precursor, regardless of whether the microsomal preparation alone, or microsomal preparation plus S $_{104}$ were used (Table 3). The pattern observed when 4,4-dimethyl[2- 3 H $_2$]monoene (structure IIIa) was used, however, was completely different, and if 4,4-dimethylcholest-8(9)-en-3 β -ol is a physiological precursor, as is very likely, then 4,4-dimethylcholest-8(14)-en-3 β -ol cannot be considered as an intermediate because in this case, a Δ^8 or Δ^7 bond does not occur until a stage after removal of the 4-methyl groups.

Cholesterol and cholest-7-enol in the presence of microsomal preparations plus S $_{104}$ were formed in relatively small amounts (3.9 and 6.2% respectively, Table 3) from 4,4-dimethyl[2- 3 H $_2$]cholest-8(14)-en-3 β -ol owing, at least in part, to the apparent difficulty of isomerization of the $\Delta^{8(14)}$ bond. On the other hand, under identical conditions, cholesterol and cholest-7-enol were synthesized in 14.6 and 17.6% yield, respectively, from dihydro[14 C]lanosterol. The corresponding values for the 4,4-dimethyl[2- 3 H $_2$]cholesta-8,14-dien-3 β -ol substrate were 19.0 and 27.9%. These values also provide good evidence for the exclusion of 4,4-dimethylcholest-8(14)-en-3 β -ol as an intermediate in the synthesis of cholesterol *de novo*, at least in mammalian liver.

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