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The Formation and Reduction of the 14,15-Double Bond in Cholesterol Biosynthesis

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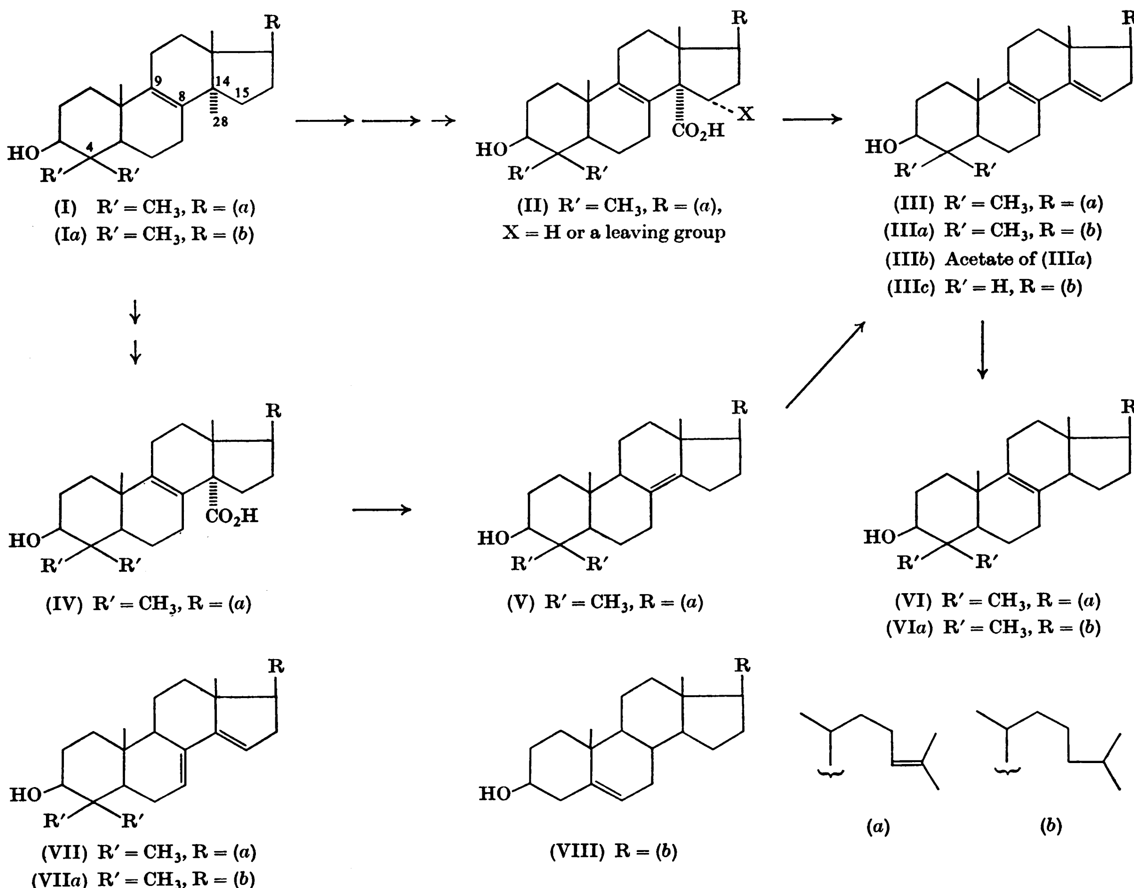
It was shown that 100μg quantities of 4,4′-dimethyl[2-$^3H_2$]cholesta-8,14-dien-3β-ol (III*a*), tritiated cholesta-8,14-dien-3β-ol, 4,4′-dimethyl[2-$^3H_2$]cholesta-7,14-dien-3β-ol, dihydro[2-$^3H_2$]lanosterol and [24-^{3}H]lanosterol were converted by a 10000g supernatant of rat liver homogenate into cholesterol in 17%, 54%, 6%, 9.5% and 24% yields respectively. From an incubation of dihydro[3α-^{3}H]lanosterol with a rat liver homogenate in the presence of a trap up to 38% of the radioactivity was found to be associated with a fraction that was unambiguously shown to be 4,4′-dimethylcholesta-8,14-dien-3β-ol. Another related compound, 4,4′-dimethylcholesta-7,14-dien-3β-ol was also shown to be equally effective in its ability to trap compound (III*a*) from an incubation of dihydro[3α-^{3}H]lanosterol. The mechanism of the further conversion of the compound (III*a*) into cholesterol occurred by the reduction of the 14,15-double bond and involved the addition of a hydrogen atom from the medium to C-15 and another from the 4-position of NADPH to C-14. Two possible mechanisms for the removal of the 14α-methyl group in sterol biosynthesis are discussed.

Recent work on sterol biosynthesis has shown that in the conversion of lanosterol (I) into cholesterol (VIII) one of the C-15 hydrogen atoms is removed (Canonica *et al.* 1968*a*; Akhtar, Watkinson, Rahimtula, Wilton & Munday, 1968, 1969*a*; Gibbons, Goad & Goodwin, 1968; Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969*b*) and at a subsequent stage is replaced by a proton from the medium (Akhtar, Rahimtula & Wilton, 1969*d*). Several mechanisms for the labilization of a C-15 hydrogen atom in cholesterol biosynthesis that have been considered suggest that this may be associated with the removal of the 14-methyl group and involve the intermediacy of a 8,14-diene system of the type (III), Scheme 1 (Canonica *et al.* 1968*a*; Akhtar *et al.* 1968, 1969*a,b*). In accordance with this proposal (Scheme 1) the conversion of 4,4′-dimethylcholesta-8,14-dien-3β-ol (IIIa, hereinafter called dimethyl-8,14-diene) into cholesterol has been demonstrated (Akhtar *et al.* 1969*a*; Canonica *et al.* 1968*b*). This paper provides evidence for the obligatory formation of dimethyl-8,14-diene (III*a*) from [3α-^{3}H]dihydrolanosterol (I*a*) and describes experiments pertinent to the mechanism for the further conversion of the compound (III*a*) into cholesterol. A part of this work has been published in preliminary reports (Watkinson & Akhtar; 1969; Akhtar *et al.* 1968*a*; see also Fiecchi *et al.* 1969).

RESULTS

Conversion of 4,4′-*dimethylcholesta*-8,14-*dien*-3β-*ol* (*IIIa*) *and related compounds into cholesterol.* The physiological intermediate in the conversion of lanosterol into cholesterol according to Scheme 1 is expected to be 4,4′-dimethylcholesta-8,14,24-trien-3β-ol (III). However, the unavailability of sterol intermediates having the 24,25-double bond necessitated the use of compounds with the saturated side chain. This approach is justified by the observations showing that enzymes participating in reactions involving the steroid nucleus are relatively insensitive to the saturation status of the side chain (Dempsey, 1965).

Radioactive dimethyl-8,14-diene (III*a*) used in the previous studies (Akhtar *et al.* 1969*a*; Canonica *et al.* 1968*b*) was labelled with tritium at undetermined positions, some of which are expected to be labilized during cholesterol biosynthesis, therefore not allowing the true estimation of the overall conversion to be made. Thus for a critical evaluation of the problem several specifically labelled steroids were required. These compounds, 4,4′-dimethyl[2-$^3H_2$]cholesta-8,14-dien-3β-ol (III*a*), dihydro[2-$^3H_2$]lanosterol, [24-^{3}H]lanosterol etc. were synthesized as described in the Experimental section and their metabolism was studied (Table 1).



Scheme 1.

The 25% conversion of lanosterol into cholesterol compared with 10% for dihydrolanosterol is noteworthy and suggests that the saturated compound (I*a*) is metabolized by the enzymes of steroid biosynthesis, but at a decreased rate (Expt. 1, Table 1). A 17% conversion of the dimethyl-8,14-diene (III*a*) compares well with the 10% conversion of its expected precursor, dihydrolanosterol, into cholesterol (Expt. 1, Table 1). The conversion of labelled lanosterol was progressively decreased by the presence of increasing amounts of unlabelled dimethyl-8,14-diene (III*a*) and vice versa (Expts. 2 and 3, Table 1). These results suggest that some of the enzymes involved in the conversion of lanosterol into cholesterol may also be employed in the conversion of the dimethyl-8,14-diene (III*a*) into cholesterol.

In view of the above results it was of interest to study the conversion of cholesta-8,14-dien-3β-ol, an analogue of the compound (III*a*), into cholesterol. Cholesta-5,7-dien-3β-ol acetate was rearranged (Fieser & Ourisson, 1953) in the presence of tritiated water to yield after hydrolysis the 8,14-diene (III*c*), which should in principle contain tritium at positions C-5, C-6, C-7 and C-15. When a sample (500μg, 4.2×10^5 c.p.m.) of the tritiated 8,14-diene (III*c*) was incubated by the method used in Table 1, the biosynthesized cholesterol had incorporated 2.3×10^5 c.p.m. or 54% of the original radioactivity. This value is minimal, however, since protons at positions C-5, C-6 and C-7 are removed during the conversion into cholesterol. In an identical control experiment carried out under anaerobic conditions only 1.5×10^3 c.p.m. (less than 0.4%) were incorporated into cholesterol. Similar results have been reported previously (Akhtar *et al.* 1968; Lutsky & Schroepfer, 1968).

Trapping of radioactivity from an incubation of dihydro[3α-^{3}H]*lanosterol* (*Ia*) *in a fraction corresponding to dimethyl*-8,14-*diene* (*IIIa*). Although

Table 1. *Conversion of dimethyl-8,14-diene (IIIa) and related sterols into cholesterol*

Cholesterol was biosynthesized from the sterols shown below with samples (15ml) of $10000g_{av.}$ rat liver homogenate, in 90min incubations at 37°C in air unless otherwise stated. The conditions were as described below, and the cholesterol was isolated and purified as its dibromide derivative.

Expt. no.	Substrate	Additions	Conversion into cholesterol (%)
1a	[2-$^3H_2$]Dimethyl-8,14-diene (IIIa), 100μg, 6.4×10^5 c.p.m.		17.2
1b	[24-^{3}H]Lanosterol, 100μg, 4.4×10^5 c.p.m.		24.0
1c	Dihydro[2-$^3H_2$]lanosterol, 100μg, 3.3×10^5 c.p.m.		9.4
2a	[2-$^3H_2$]Dimethyl-8,14-diene (IIIa), 52μg, 3.3×10^5 c.p.m.		10.1
2b	[2-$^3H_2$]Dimethyl-8,14-diene (IIIa), 52μg, 3.3×10^5 c.p.m.	Lanosterol, 100μg	5.3
2c	[2-$^3H_2$]Dimethyl-8,14-diene (IIIa), 52μg, 3.3×10^5 c.p.m.	Lanosterol, 500μg	1.6
2d	[2-$^3H_2$]Dimethyl-8,14-diene (IIIa), 52μg, 3.3×10^5 c.p.m.	N_2	0.14
3a	[24-^{3}H]Lanosterol, 50μg, 2.2×10^5 c.p.m.		7.6
3b	[24-^{3}H]Lanosterol, 50μg, 2.2×10^5 c.p.m.	Dimethyl-8,14-diene, 100μg (IIIa)	5.1
3c	[24-^{3}H]Lanosterol, 50μg, 2.2×10^5 c.p.m.	Dimethyl-8,14-diene, 1mg (IIIa)	1.6
3d	[24-^{3}H]Lanosterol, 50μg, 2.2×10^5 c.p.m.	N_2	0.26
4a	[2-$^3H_2$]Dimethyl-8,14-diene (IIIa), 100μg, 6.4×10^5 c.p.m.		12.7
4b	[2-$^3H_2$]Dimethyl-7,14-diene (VIIa), 100μg, 1×10^6 c.p.m.		5.9

the experiments described above indicate that the dimethyl-8,14-diene (III*a*) is converted into cholesterol along the normal biosynthetic pathway, a more definite proof of the involvement of this diene (III*a*) in cholesterol biosynthesis was sought by making use of the technique of radioactive trapping.

Samples of dihydro[3α-^{3}H]lanosterol (I*a*; 100μg, 1.7×10^6c.p.m.) were incubated under aerobic conditions with a $10000g_{av.}$ supernatant of rat liver homogenate in the presence of a trap of dimethyl-8,14-diene (III*a*; 2mg). After 90min, the incubations were terminated by the addition of methanolic 10% potassium hydroxide, and the non-saponifiable sterols were extracted and acetylated overnight. From one such incubation the acetylated material was diluted with carrier (III*b*; 23mg) and purified chromatographically by running on two successive silver nitrate-impregnated silica gel HF_{254} plates. The band (R_F 0.41) corresponding to the compound (III*b*) was eluted, evaporated to dryness and the product crystallized from diethyl ether–methanol, and had 2.5×10^4c.p.m./mg. Since a total of 25mg of carrier was added (2mg in the trap, 23mg as carrier), this represents an overall conversion of 6.4×10^5c.p.m., or 38%, from dihydrolanosterol (I*a*) into dimethyl-8,14-diene (III*a*). A sample of the above material (4mg, 1×10^5c.p.m.) was diluted with carrier (III*b*; 34.5mg) and crystallized five times from diethyl ether–methanol. At each stage a sample was vacuum-dried and counted, and the values of the specific radioactivities obtained (c.p.m./mmol) are: first crystallization 1.22×10^6; second crystallization 1.21×10^6; third crystallization 1.20×10^6; fourth crystallization 1.21×10^6; fifth crystallization 1.21×10^6.

The absolute constancy of these specific radioactivity measurements provides strong evidence that the trapped radioactivity was associated with the acetate (III*b*). This was further confirmed by the following experiment. A sample of the acetate (III*b*; 5mg, 1.17×10^7c.p.m./mmol) from the trapping experiment was catalytically hydrogenated with Raney nickel in ethanol to yield the reduced derivative (VI*b*; 1.13×10^7c.p.m./mmol) showing full retention of radioactivity. On chromatographic analysis on a silver nitrate-impregnated plate the compound (VII*b*) was shown to be more than 98.3% radiochemically pure. A table of the R_F values of related monoenes and dienes is shown in Table 2. In view of the similar chromatographic

Table 2. *R_F values of sterol acetates*

	R_F
Lanosterol acetate	0.55
Dihydrolanosterol acetate	0.61
4,4′-Dimethylcholest-5-en-3β-ol acetate	0.58
4,4′-Dimethylcholest-7-en-3β-ol acetate	0.54
4,4′-Dimethylcholest-8-en-3β-ol acetate	0.54
4,4′-Dimethylcholest-8(14)-en-3β-ol acetate	0.55
4,4′-Dimethylcholesta-8,14-dien-3β-ol acetate	0.41
4,4′-Dimethylcholesta-7,14-dien-3β-ol acetate	0.41
4,4′-Dimethylcholesta-5,7-dien-3β-ol acetate	0.53
4,4′-Dimethylcholesta-6,8(14)-dien-3β-ol acetate	0.46

Table 3. *Recrystallization of* 4,4′-*dimethylcholesta*-8,14-*dien*-3β-*ol acetate* (*IIIb*) *in the presence of trace amounts of radioactive* 4,4′-*dimethylcholesta*-7,14-*dien*-3β-*ol acetate*

Two similar experiments were set up, one containing 200mg of non-radioactive diene (III*b*) together with 4,4′-dimethyl[2-$^3H_2$]cholesta-7,14-dien-3β-ol acetate (37μg, 4.5×10^5c.p.m.), and the other containing 100mg of the 8,14-diene (III*b*) and 30μg (3.6×10^5c.p.m.) of the radioactive 7,14-diene. Each preparation was recrystallized five times from diethyl ether–methanol and the specific activity measured at each step.

No. of crystallizations	Expt. 1 (c.p.m./mg)	Loss of radioactivity (%)	Expt. 2 (c.p.m./mg)	Loss of radioactivity (%)
1	2040	0	3580	0
2	1880	7.8	3240	9.5
3	1730	15.2	3060	14.5
4	1535	24.8	2600	27.5
5	1387	32.0	2210	38.0

properties of steroids containing the 8,14- and 7,14-diene systems (Table 2), further evidence that the radioactivity trapped from the incubation of dihydrolanosterol was in fact associated with the dimethyl-8,14-diene (III*a*) was sought.

Labelled 4,4′-dimethylcholesta-7,14-dien-3β-ol acetate (VII*b*) was prepared as described in the Experimental section and two samples of this diene (37μg, 4.5×10^5c.p.m.; 30μg, 3.6×10^5c.p.m.) were mixed with 200mg and 100mg respectively of unlabelled dimethyl-8,14-diene acetate (III*b*) and each sample was then recrystallized five times from diethyl ether–methanol. A sample was taken at each stage of the crystallizations and counted. The results in Table 3 show a linear drop in specific activity of up to 30–40% over the five crystallization steps. Since no such drop in the radioactivity was noted when the diene compound trapped from the incubation of dihydrolanosterol was co-crystallized with dimethyl-8,14-diene acetate (III*b*), it must be concluded that the trapped radioactive intermediate was dimethyl-8,14-diene (III*a*) and not dimethyl-7,14-diene (VII*a*).

Biological conversion of dimethyl-7,14-*diene* (*VIIa*) *into cholesterol.* The availability of radioactive dimethyl-7,14-diene (VII*a*) from the above experiment was exploited to test this compound as a precursor of cholesterol. Accordingly, samples (100μg) of this diene (VII*a*) and dimethyl-8,14-diene (III*a*) were separately incubated with portions of a rat liver homogenate. The 6% conversion (Table 1) of dimethyl-7,14-diene (VII*a*) into cholesterol compared with the 13% conversion of dimethyl-8,14-diene (III*a*) indicates that the former diene (VII*a*) is about 50% as effective a precursor of cholesterol as the latter (III*a*).

Having established that (VII*a*) is capable of being converted into cholesterol, its ability to trap radioactivity from an incubation of dihydro[3α-^{3}H]-lanosterol (I*a*) was also investigated. Dihydro-[3α-^{3}H]lanosterol was aerobically incubated with portions of a rat liver homogenate in the presence of (i) a trap of dimethyl-8,14-diene (III*a*; 2mg), (ii) a trap of dimethyl-7,14-diene (VII*a*; 2mg), (iii) no trap. The incubations were terminated after 90min and the total sterols extracted and acetylated overnight and then diluted with 50mg of carrier (III*b*). The material from each experiment was processed as above and then crystallized five times from diethyl ether–methanol and a sample counted at each stage as before, the results being summarized in Table 4. From these it can be seen that both dienes (III*a* and VII*a*) are equally effective in their ability to trap radioactivity in high yield. Furthermore, in

Table 4. *Comparison of the effectiveness of* 7,14- *and* 8,14-*dienes as trapping agents*

Aerobic incubations of dihydro[3α-^{3}H]lanosterol (100 μg, 1.7×10^{6} c.p.m.) were carried out with 10000$g_{av.}$ supernatant of rat liver homogenate in the presence of (i) a trap (2 mg) of 4,4′-dimethylcholesta-8,14-dien-3β-ol, (ii) a trap (2 mg) of 4,4′-dimethylcholesta-7,14-dien-3β-ol, (iii) no trap. The sterols were extracted, acetylated, mixed with 50 mg of non-radioactive (III*a*) purified by preparative layer chromatography and crystallized five times each. The specific activities of the three products are shown below at each stage of the crystallization.

No. of crystallizations	Specific radioactivity (c.p.m./mg)		
	8,14-Diene trap	7,14-Diene trap	No trap
1	11042	11358	1221
2	11313	10967	981
3	10495	10690	935
4	11875	12180	954
5	11343	11225	874
Total c.p.m trapped	561000	564000	46500
Percentage trapped	33.0	33.2	2.7

both cases, since the specific activity of the acetate (III*a*) was not lowered over the crystallization steps, the radioactivity that had been trapped was associated with dimethyl-8,14-diene (III*a*). These two experiments also show that although capable of being converted into cholesterol, the diene (VII*a*) is not a true intermediate in cholesterol biosynthesis, since it could not be trapped from an incubation of dihydro[3α-^{3}H]lanosterol. However, since this diene (VII*a*) was equally effective as dimethyl-8,14-diene (III*a*) in the trapping experiments, it is apparent that (VII*a*) can act as an efficient substitute for (III*a*) in enzymic reactions.

Enzymic reduction of the 14,15-*double bond of dimethyl*-8,14-*diene* (IIIa). The trapping experiments described above demonstrate the presence in rat liver of an enzyme system capable of generating dimethyl-8,14-diene (III*a*) from dihydrolanosterol. It is now shown that there is also present in rat liver an enzyme system capable of converting this diene (III*a*) into the reduced derivative (VI*a*). The mechanism of the reduction of the 14,15-double bond in the conversion (III*a*) → (VI*a*) was studied as follows. 4,4′-Dimethylcholest-8-en-3β-ol (VI*a*) was biosynthesized from the diene (III*a*) in the presence of either tritiated water or [4-$^{3}H_2$]-NADPH. The incubation was carried out with washed microsomes to prevent randomization of the 4-position hydrogen atoms of the pyridine nucleotide, and was performed under anaerobic conditions to prevent any further conversion of the reduced derivative (VI*a*) so formed. The diene (III*a*, 500 μg) was incubated under an atmosphere of purified nitrogen with washed microsomes (10 ml) in the presence of [4-$^{3}H_2$]NADPH (3 mg; 9.9×10^{6} c.p.m.). The total sterols were extracted and acetylated overnight and then diluted with carrier (VI*b*; 35 mg) and purified by running on silica gel HF_{254}–10% silver nitrate preparative layer plates. The band (R_F 0.54) was eluted and on crystallization had a specific activity of 2082 c.p.m./mg. This was rearranged with platinum oxide and hydrogen to give 4,4′-dimethylcholest-8(14)-en-3β-ol acetate (52.5 c.p.m./mg), which was shown to have lost 97.5% of its radioactivity. Only the C-14 hydrogen is lost by this rearrangement, thus indicating that all the radioactivity has been associated with this position.

In another experiment, the diene (III*a*; 500 μg) was incubated under nitrogen with washed microsomes (10 ml) containing tritiated water (0.25 ml, 50 mCi). $NADP^{+}$ and a NADPH-regenerating system were added and the experiment was continued as described above. The purified (VI*b*) (306 c.p.m./mg) was rearranged to 4,4′-dimethylcholest-8(14)-en-3β-ol acetate (313 c.p.m./mg) with full retention of radioactivity indicating that the tritium atom was resident at C-15. This proves that in the reduction of the 14,15-double bond of dimethyl-8,14-diene the C-14 proton is derived from NADPH and indicates that the C-15 proton originates from the medium. An unambiguous demonstration that the tritiated water-derived radioactivity is in fact located at C-15 has been established in the reduction of (IIIc) (Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969*c*).

DISCUSSION

The efficient conversion of dimethyl-8,14-diene (III*a*) into cholesterol and its formation from dihydrolanosterol in up to 38% yield established the intermediary role of 8,14-dienes of the type (III) in sterol biosynthesis. The trapping experiments further endorse the earlier view of Gautschi & Bloch (1957, 1958) that in the hepatic biosynthesis of cholesterol from lanosterol it is the 14α-methyl group that is first removed. The further conversion

of dimethyl-8,14-diene (III*a*) into cholesterol occurs through the reduction of the 14,15-double bond involving the addition of a hydrogen from NADPH to C-14 and another from a proton source to C-15. This orientation of addition is in accordance with the predictions of Wilton, Munday, Skinner & Akhtar (1968). The stereochemical aspects of the conversion of structures of the type (III) into (VI) showing a *trans* addition have been examined (Akhtar *et al.* 1969*d*; Caspi, Ramm & Gain, 1969). The enzyme participating in the reduction of the 14,15-double bond is present in the microsomal fraction and shows a relatively broad specificity since it is effective with substrates containing the second double bond in either the 7,8- (as in VII) or the 8,9- (as in III) position. The corresponding compounds lacking the 4,4′-dimethyl substituent are also metabolized by the enzyme though at a somewhat diminished efficiency (see Wilton, Watkinson & Akhtar, 1970).

The process through which the dimethyl-8,14-diene (III*a*) is formed from dihydrolanosterol is not yet established, though two broad mechanistic pathways for the conversion may be considered. The first type of mechanism rationalizes the participation of the C-15 hydrogen atom by a process in which the cleavage of the C-14–C-28 bond occurs concomitantly with the elimination of a 15α- substituent (II → III, Scheme 1) as discussed previously (Akhtar *et al.* 1968, 1969*a*,*b*; Canonica *et al.* 1968*a*,*b*). The second type of mechanism envisages the cleavage of the C-14–C-28 bond occurring by the process (IV) → (V). The resulting 8,14-ene (V) presumably cannot be directly isomerized to the 8,9-ene (VI) and this is achieved indirectly by the sequence (V) → (III) → (VI). Further work is required to differentiate between the two types of mechanisms.

EXPERIMENTAL

Materials were obtained, radioactivity was measured, [4-$^3H_2$]NADPH was prepared and incubations with either a $10000g_{av.}$ supernatant of rat liver homogenate or the $105000g_{av.}$ microsomal pellet were performed as described previously (Wilton *et al.* 1968). [24-^{3}H]Lanosterol was prepared according to the method of Akhtar, Hunt & Parvez (1967). Cholesterol was isolated and purified as described by Wilton *et al.* (1968). All other sterols were similarly isolated and then acetylated overnight in pyridine (3ml) and acetic anhydride (1ml). The chromatographic systems used for the purification and analysis of these sterol acetates were either preparative layer (20cm×10cm, 1mm-thick) silica gel HF_{254}–10% $AgNO_3$ plates, or analytical layer silica gel GH_{254}–10% $AgNO_3$ plates developed in light petroleum (b.p. 60–80°C)–benzene (3:7, v/v). R_F values are displayed in Table 2.

Preparation of Raney nickel capable of reducing the 14,15-double bond of 4,4′-dimethylcholesta-8,14-dien-3β-ol acetate. Sodium hydroxide (20g) was dissolved in water (40ml) and to this rapidly stirred solution, cooled in ice, nickel–aluminium alloy (10g; 1:1, w/w) was gradually added to maintain the temperature between 70° and 75°C. At 5min after the last addition of alloy, the aqueous layer was decanted off and the Raney nickel was washed rapidly with water (5×50ml) and ethanol (3×50ml). The catalyst could be stored for up to one day at −15°C under ethanol and still retain its catalytic properties; normally, however, it was used immediately. Total time of preparation should not exceed 20min.

Preparation of 4,4′-dimethylcholest-8-en-3β-ol. 4,4′-Dimethylcholesta-8,14-dien-3β-ol acetate (700mg) was mixed with ethanol (150ml), a sample of the Raney nickel (about 500mg) prepared as above was added, and the diene was hydrogenated at room temperature and atmospheric pressure for 2h. After this time the catalyst was removed by filtration through kieselguhr and the solution was evaporated to dryness. The solid product so obtained showed no diene absorption in u.v., and was homogeneous on t.l.c. analysis. Two crystallizations from diethyl ether–methanol yielded pure 4,4′-dimethylcholest-8-en-3β-ol acetate, 410mg, m.p. 122–125°C, $[\alpha]_D$ +35.7°. [Gautschi & Bloch (1958) quote m.p. 122–124°C, $[\alpha]_D$ +37°.] A sample of this (200mg) was reduced with excess of $LiAlH_4$ in dry diethyl ether and the product prepared in the usual way and crystallized from diethyl ether–methanol to yield pure 4,4′-dimethylcholest-8-en-3β-ol, 135mg (m.p. 159–160°C).

Preparation of 4,4′-dimethyl[2-$^3H_2$]*cholesta-8,14-dien-3β-ol.* 4,4′-Dimethylcholesta-5,7-dien-3-one (400mg) was heated at 100° in a sealed tube for 2h with dioxan (10ml), KOH (150mg) and tritiated water (0.1ml, 100mCi). The mixture was then diluted with water (100ml) and extracted with 3×25ml of light petroleum (b.p. 40–60°C)–diethyl ether (1:1, v/v). The ethereal extracts were pooled and washed with 10×25ml of water and 2×25ml of saturated NaCl soln., dried over anhydrous Na_2SO_4 and evaporated to give a solid product. This was crystallized once from diethyl ether–methanol and the product reduced with excess of $LiAlH_4$ in sodium-dried diethyl ether to give after crystallization from diethyl ether–methanol 4,4′-dimethyl[2-$^3H_2$]cholesta-5,7-dien-3β-ol (300mg, m.p. 139–141°C). This was dissolved in a mixture of 95% ethanol (6ml), benzene (0.75ml) and 36% (w/v) HCl (1.5ml) and the mixture boiled under reflux for 3h. Water (10ml) was added and the mixture extracted with 3×25ml of light petroleum (b.p. 40–60°C)–diethyl ether (1:1, v/v). The ethereal extracts were pooled, washed with 3×25ml of water and 1×20ml of saturated NaCl soln., dried over anhydrous Na_2SO_4 and evaporated to give a solid product. This was crystallized three times from diethyl ether–methanol to yield 4,4′-dimethyl[2-$^3H_2$]cholesta-8,14-dien-3β-ol (250mg). A sample of this (50mg) was retained and the rest was acetylated overnight in a mixture of pyridine (6ml) and acetic anhydride (2ml) to yield, after the usual procedure and two crystallizations from diethyl ether–methanol, pure 4,4′-dimethylcholesta-8,14-dien-3β-ol acetate, 200mg, 6.7×10^6 c.p.m./mg, m.p. 151–152°C, $[\alpha]_D$ −22°, $\epsilon = 18630$. [Gautschi & Bloch (1958) quote m.p. 151–153°C, $[\alpha]_D$ −23°, ϵ 18000.]

Preparation of 4,4′-dimethyl[2-$^3H_2$]*cholesta-7,14-dien-3β-ol.* 4,4′-Dimethyl[2-$^3H_2$]cholesta-5,7-dien-3β-ol acetate (250mg), obtained as above, in chloroform (20ml) was cooled to −30° and maintained at this temperature for 2h whilst dry HCl gas was passed through (Knight,

Klein & Szczepanik, 1966). The resulting mixture was immediately poured into rapidly stirred ice-cold saturated $NaHCO_3$ soln. (100ml), which was then extracted with 3×25ml of chloroform. The chloroform extract was then washed with water (3×25ml) and saturated NaCl soln. (1×20ml), dried over anhydrous Na_2SO_4 and evaporated to give a solid product which was crystallized once from diethyl ether–methanol to give pure 4,4′-dimethyl[2-$^3H_2$]-cholesta-7,14-dien-3β-ol acetate, 205mg, 1.03×10^7 c.p.m./mg, m.p. 117–119°C, $[\alpha]_D$ −127°, $\lambda_{max.}$ 243nm ($\epsilon = 9900$).

Preparation of labelled dihydrolanosterol. Dihydro-[2-$^3H_2$]lanosterol was prepared from the corresponding 3-ketone by the method used for the preparation of 4,4′-dimethyl[2-$^3H_2$]cholesta-5,7-dien-3β-ol. Dihydro-[3α-^{3}H]lanosterol was obtained by the NaB^3H_4 reduction of the 3-ketone.

Conversion of biosynthetic 4,4′-*dimethylcholest*-8-*en*-3β-*ol acetate into* 4,4′-*dimethylcholest*-8(14)-*en*-3β-*ol acetate.* After pre-reduction of platinum oxide (50mg) in ethyl acetate (20ml), 4,4′-dimethylcholest-8-en-3β-ol acetate and a catalytic quantity of perchloric acid in ethyl acetate (10ml) were added. The hydrogenation was allowed to proceed with rapid stirring at room temperature and atmospheric pressure for 2h. The product was filtered through kieselguhr and the filtrate evaporated to dryness. The solid product was crystallized from diethyl ether–methanol to yield 4,4′-dimethylcholest-8(14)-en-3β-ol acetate (22mg, m.p. 115–116°C).

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REFERENCES

Akhtar, M., Hunt, P. F. & Parvez, M. A. (1967). *Biochem. J.* **103**, 616.

Akhtar, M., Rahimtula, A. D., Watkinson, I. A., Wilton, D. C. & Munday, K. A. (1969*b*). *Eur. J. Biochem.* **9**, 107.

Akhtar, M., Rahimtula, A. D., Watkinson, I. A., Wilton, D. C. & Munday, K. A. (1969*c*). *Chem. Commun.* p. 149.

Akhtar, M., Rahimtula, A. D. & Wilton, D. C. (1969*d*). *Biochem. J.* **114**, 801.

Akhtar, M., Watkinson, I. A., Rahimtula, A. D., Wilton, D. C. & Munday, K. A. (1968). *Chem. Commun.* p. 1406.

Akhtar, M., Watkinson, I. A., Rahimtula, A. D., Wilton, D. C. & Munday, K. A. (1969*a*). *Biochem. J.* **111**, 757.

Canonica, L., Fiecchi, A., Kienle, M. G., Scala, A., Galli, G., Paoletti, E. G. & Paoletti, R. (1968*a*). *J. Am. chem. Soc.* **90**, 3597.

Canonica, L., Fiecchi, A., Kienle, M. G., Scala, A., Galli, G., Paoletti, E. G. & Paoletti, R. (1968*b*). *J. Am. chem. Soc.* **90**, 6532.

Caspi, E., Ramm, P. J. & Gain, R. E. (1969). *J. Am. chem. Soc.* **91**, 4012.

Dempsey, M. (1965). *J. biol. Chem.* **240**, 4176.

Fiecchi, A., Canonica, L., Scala, A., Cattabeni, F., Grossi Paoletti, E. & Paoletti, R. (1969). *Life Sci.* **8**, 629.

Fieser, L. F. & Ourisson, J. (1953). *J. Am. chem. Soc.* **75**, 4404.

Gautschi, F. & Bloch, K. (1957). *J. Am. chem. Soc.* **79**, 684.

Gautschi, F. & Bloch, K. (1958). *J. biol. Chem.* **233**, 1343.

Gibbons, G. F., Goad, L. J. & Goodwin, T. W. (1968). *Chem. Commun.* p. 1458.

Knight, J. C., Klein, P. D. & Szczepanik, P. A. (1966). *J. biol. Chem.* **241**, 1502.

Lutsky, B. N. & Schroepfer, G. J. (1968). *Biochem. biophys. Res. Commun.* **33**, 492.

Watkinson, I. A. & Akhtar, M. (1969). *Chem. Commun.* p. 206.

Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968). *Biochem. J.* **106**, 803.

Wilton, D. C., Watkinson, I. A. & Akhtar, M. (1970). *Biochem. J.* **119**, 673.