Biosynthesis of Cholesterol and Lanosterol in Isolated Dog Hepatocytes: Incorporation of [1,2-$^{13}C_2$]- and [2-$^{13}C^2H_3$]-Acetate, and [1-^{13}C]Acetate and [1-$^2H_2$]Ethanol

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Since isolated dog hepatocytes have been shown to exhibit potent activity for the synthesis of cholesterol from acetic acid, an analysis of cholesterol and lanosterol biosynthesized from [1,2-$^{13}C_2$]- or [2-$^{13}C^2H_3$]acetate in fresh isolated hepatocytes has been attempted in order to clarify the incorporation and distribution of the acetate carbon and hydrogen into these sterols. Lanosterol was obtained by adding SSF-109, a sterol biosynthesis inhibitor, to the hepatocytes suspension. ^{13}C NMR spectroscopic analysis of the [^{13}C]- and [$^{13}C^2H$]-labelling patterns of these compounds confirmed the following: (i) the involvement of two 1,2-hydride shifts, 20-H from C-17 and 17-H from C-13; (ii) two 1,2-methyl migrations, 13-methyl group (C-18) from C-14 and 14-methyl group (C-32) from C-8 in the cyclization of 2,3-oxidosqualene to form lanosterol; and (iii) stereospecific hydrogenation from the *Si*-face at C-25 in the conversion of lanosterol into cholesterol. The proton-decoupled ^{13}C–^1H COSY spectrum confirmed the stereospecific hydrogen attack at C-7, C-15 and C-24. The deuterium atoms of [1-$^2H_2$]ethanol were incorporated into lanosterol at C-2, C-6, C-11, C-12, C-16 and C-23, which arise from C-5 of mevalonic acid.

The biosynthesis of cholesterol is based on the conjugation of acetyl-CoA (active acetate), the two carbon atoms of acetate being considered as the building blocks of sterols.[1] The multi-step synthesis takes place as follows. First mevalonate is biosynthesized, after which condensation of the isoprenoid units gives squalene.[2] Lanosterol, the first cyclic intermediate from squalene, is converted into cholesterol successively *via* three demethylations from the 4α-, 4β- and 14α-positions, a migration of the 8(9) double bond to form the 5(6) double bond, and a reduction of the 24(25) double bond.[2] Using rats, Cornforth, Gore and Popják[3] examined the distribution patterns of C-1 and C-2 carbons of acetate in cholesterol. We have also shown the incorporation patterns of [2-$^{13}C^2H_3$]- and [1,2-$^{13}C_2$]-acetate into various sterols, for example, ergosterol in yeast[4] and phytosterols in tissue cultures of higher plants.[4]

To confirm the previous findings on distribution of carbon and hydrogen atoms of acetate in cholesterol, we used isolated dog hepatocytes and examined the [^{13}C]- and [$^{13}C^2H$]-labelling patterns of cholesterol and lanosterol using [1,2-$^{13}C_2$]- and [2-$^{13}C^2H_3$]-acetate as source of the sterols. Stereospecific hydrogen attacks at C-7 and C-15 of cholesterol were determined by the proton-decoupled ^{13}C–^1H COSY spectrum. The incorporation patterns of [1-$^2H_2$]ethanol in the presence of [1-^{13}C]acetate into lanosterol was also explored. Thus, use of stable isotopes, NMR spectroscopy and isolated hepatocytes, in combination, afforded assignment of all the carbon and hydrogen atoms originated from acetate. The results are consistent with the previous findings on cholesterol biosynthesis.[2]

Results and Discussion

Incorporation of [1,2-$^{13}C_2$]- *and* [2-$^{13}C^2H_3$]-*Acetate into Cholesterol.*—In order to study the ability of cholesterol biosynthesis in dog liver, isolated hepatocytes, obtained from beagle dogs, were incubated with [1-^{14}C]acetate and [2-^{14}C]-MVA. Canine hepatocytes showed considerably higher activity in synthesizing cholesterol than did rat hepatocytes[5] (Table 1).

Table 1 Comparison of the incorporation of [1-^{14}C]acetate and [2-^{14}C]mevalonate into cholesterol by rat and dog hepatocytes

		Incorporation into cholesterol[a]	
Animal	n[b]	[1-^{14}C]Acetate	[2-^{14}C]Mevalonate
Rat	3	4772 ± 330	6385 ± 468
Dog	5	47 751 ± 7714	15 796 ± 4360

Values are mean ± S.E. [a] Dpm h^{-1} 10^7 $cells^{-1}$. [b] No. of heads.

Isolated dog hepatocytes were incubated with [1,2-$^{13}C_2$]- or [2-$^{13}C^2H_3$]-acetate added in three portions. After 3 h incubation, [^{13}C]-labelled cholesterol **3** from [1,2-$^{13}C_2$]acetate, and [$^{13}C^2H$]-labelled cholesterol **6** from [2-$^{13}C^2H_3$]acetate were isolated. The labelling patterns of **3** are shown in Table 2. Nine carbons, C-1, C-4, C-7, C-8, C-14, C-15, C-18, C-22 and C-26 were observed as labelled singlets and nine pairs of carbons, C-2–C-3, C-5–C-6, C-9–C-11, C-10–C-19, C-12–C-13, C-16–C-17, C-20–C-21, C-23–C-24 and C-25–C-27, were found to be labelled doublets (intraunit coupling of [1,2-$^{13}C_2$]acetate unit). The labelled singlet signals, C-14 and C-18, confirmed a migration of the methyl group (C-18) from C-14 to C-13, which was originally demonstrated by Popják *et al.*[6]

The signal assignments of the two methyl groups at C-25, C-26 at δ_C 22.57 (the *pro-R* methyl group) and C-27 at δ_C 22.83 (the *pro-S* methyl group) have been established previously by preparing (25-*R*)- and (25-*S*)-[26-^2H]cholesterol.[7] Based on these assignments, the labelled singlet C-26 originating from C-2 of MVA and the labelled doublet C-27 originating from C-6

† Deceased on 14th April, 1991.

Table 2 ^{13}C NMR spectroscopic data of cholesterol **3** and **6** biosynthesized from [1,2-$^{13}C_2$]acetate and [2-$^{13}C^2H_3$]acetate, respectively, in isolated dog hepatocytes

		3			**6**			
Carbon	δ_C	s/d[a]	J_{CC}	(J_{CC})[b]	$^1\Delta\delta^{13}C^2H_n$	$^1\Delta\delta_{C^2H_1}$	$^1\Delta\delta_{C^2H_2}$	$^1\Delta\delta_{C^2H_3}$
C-1	37.27	s		(34)	$^{13}C^2H_2$	−0.43 −0.37	−0.83	
C-2	31.68	d	36		—			
C-3	71.83	d	36	(36)	—			
C-4	42.32	s		(39)	—			
C-5	140.76	d	72	(39)	—			
C-6	121.72	d	72		—			
C-7	31.92	s		(37)	$^{13}C^2H_1{}^1H_1$	−0.36		
C-8	31.92	s		(37)	—			
C-9	50.15	d	35	(35)	—			
C-10	36.52	d	*c*		—			
C-11	21.10	d	35	(35)	—			
C-12	39.79	d	33		—			
C-13	42.33	d	33		$^{13}C(\beta\text{-}^2H)$	(−0.08)[d]		
C-14	56.77	s		(34)	—			
C-15	24.30	s		(34)	$^{13}C^2H_1{}^1H_1$	−0.34		
C-16	28.25	d	33		—			
C-17	56.16	d	33		$^{13}C(\beta\text{-}^2H)$	(−0.11)[d]		
C-18	11.87	s		(36)	$^{13}C^2H_3$	−0.29	−0.57	−0.86
C-19	19.40	d	35		$^{13}C^2H_3$	−0.29	−0.59	−0.87
C-20	35.79	d	36		—			
C-21	18.73	d	36		$^{13}C^2H_3$	−0.30	−0.61	−0.93
C-22	36.20	s			$^{13}C^2H_2$	−0.47	−0.89	
C-23	23.83	d	35		—			
C-24	39.52	d	35		$^{13}C^2H_1{}^1H_1$	−0.49		
C-25	28.03	d	35	(35)	—			
C-26	22.57	s		(35)	$^{13}C^2H_2{}^1H_1$	−0.31	−0.63	
C-27	22.83	d	35		$^{13}C^2H_3$	−0.31	−0.63	−0.94

[a] s: Labelled singlet and d: labelled doublet. [b] Interunit coupling constant (Hz). [c] Not observed because of signal overlapping. [d] β-Deuterium isotope shift ($^2\Delta\delta_{C^2H}$).

of MVA were observed. These results confirmed that a hydrogenation at C-25 of the 24(25) double bond in lanosterol **2** (*vide infra*) took place stereospecifically from the *Si*-face in agreement with the previous results from rats reported by Popják *et al.*[6] The same stereospecific hydrogenation at C-25 was observed in steroidal sapogenins[8] in higher plant cells and dihydrobrassicasterol,[9] which bears a 24β-methyl group. On the other hand, the opposite stereospecificity at C-25 was found in plant systems when 24α-ethylsterols,[10] 24β-ethylsterols[10] and 24α-methylsterol[9] were formed. The same was true for ergosterol in yeast[9] and *Claviceps paspari*,[11] which has a 24β-methyl group.

The labelling patterns of cholesterol **6** were determined by ^{13}C-{^1H}{^2H}NMR spectroscopy and are shown in Table 2. The methyl groups, C-18, C-19, C-21 and C-27, were labelled as $^{13}C^2H_3$ but C-26, the *pro-R* methyl group at C-25, was labelled as $^{13}C^2H_2{}^1H$ which indicates this C-26 carbon arises from C-2 of MVA. These results confirmed the stereospecificity of the hydrogen attack from the *Si*-face at C-25 of lanosterol **5** as described above. The β-deuterium isotope shifted signals observed at C-13 ($^2\Delta\delta_C$ −0.08) and at C-17 ($^2\Delta\delta_C$ −0.11), which were confirmed by a ^{13}C-{^1H}NMR spectrum, indicate the 1,2-deuterium migrations from C-13 to C-17 and from C-17 to C-20, respectively.*

The methylene groups (C-7 and C-15) were labelled as $^{13}C^2H^1H$. These results agree with the extrusion of two hydrogen atoms from C-7 and C-15, during the double bond migration to form the 5(6) double bond and during 14α-methyl demethylation, respectively, and then a hydrogen addition at each position.[13]

Recently, Vederas *et al.* developed a new method: deuterium-decoupled ^{13}C–^1H COSY, for studying the stereospecificity of reduction without chemical degradation.[14] In order to study the stereospecificity of hydrogen addition at the C-7 and C-15, and also reduction of the 24(25) double bond, we determined the proton-decoupled ^{13}C–^1H COSY spectrum[15] of **6**. The assignment of the α- and β-hydrogen signals were made based on the homonuclear two dimensional *J*-resolved spectrum[16] and confirmed by the observation of NOE between 6-H and 7β-H in the ROESY spectrum[17] and between 14-H and 15α-H in the NOESY spectrum.[18] The assignments agreed with the results on similar compounds reported by Kirk *et al.*[19a] and Hayamizu *et al.*[19b]

Regions of the proton-decoupled ^{13}C–^1H COSY spectrum of **6** are shown in Fig. 1. Cross peaks appeared between C-7 (δ_C 31.92) and two hydrogens at C-7 [δ_H 1.474 (7α-H) and δ_H 1.973 (7β-H)]. A deuterium shifted signal (B) was observed only between C-7 and 7α-H ($^1\Delta\delta_C$ −0.36 and $^2\Delta\delta_H$ −0.019) indicating that a deuterium atom is located at the 7β-position. Cross peaks appeared between C-15 (δ_C 24.30) and two hydrogens at C-15 [δ_H 1.070 (15β-H) and δ_H 1.575 (15α-H)]. A deuterium shifted signal (A) was observed between C-15 and 15β-H ($^1\Delta\delta_C$ −0.34 and $^2\Delta\delta_H$ −0.023) indicating that a deuterium atom was retained at the 15α-position. These results agree well with the stereospecificity reported by Caspi[13] that the 2-*pro-R* hydrogen atom of MVA takes the 7β- and 15α-positions in cholesterol. Kienle *et al.*[20] and Caspi *et al.*[21] also reported that a hydrogen attacks at C-24 from the *Si*-face of the 24(25) double bond of lanosterol and 24-H of lanosterol is retained at the 24-*pro-R* position of cholesterol. The cross peaks appeared between C-24 (δ_C 39.52) and two 24-hydrogens (δ_H 1.092 and 1.143) and the higher field signal was accompanied by

* The deuterium atom at C-20 migrated from C-17 was confirmed on the C-21 signal as the interunit β-deuterium isotope shifted signals ($^2\Delta\delta_C$ −0.12 to −0.16) which were observed by maximum entropy Fourier spectral deconvolution processing.[12]

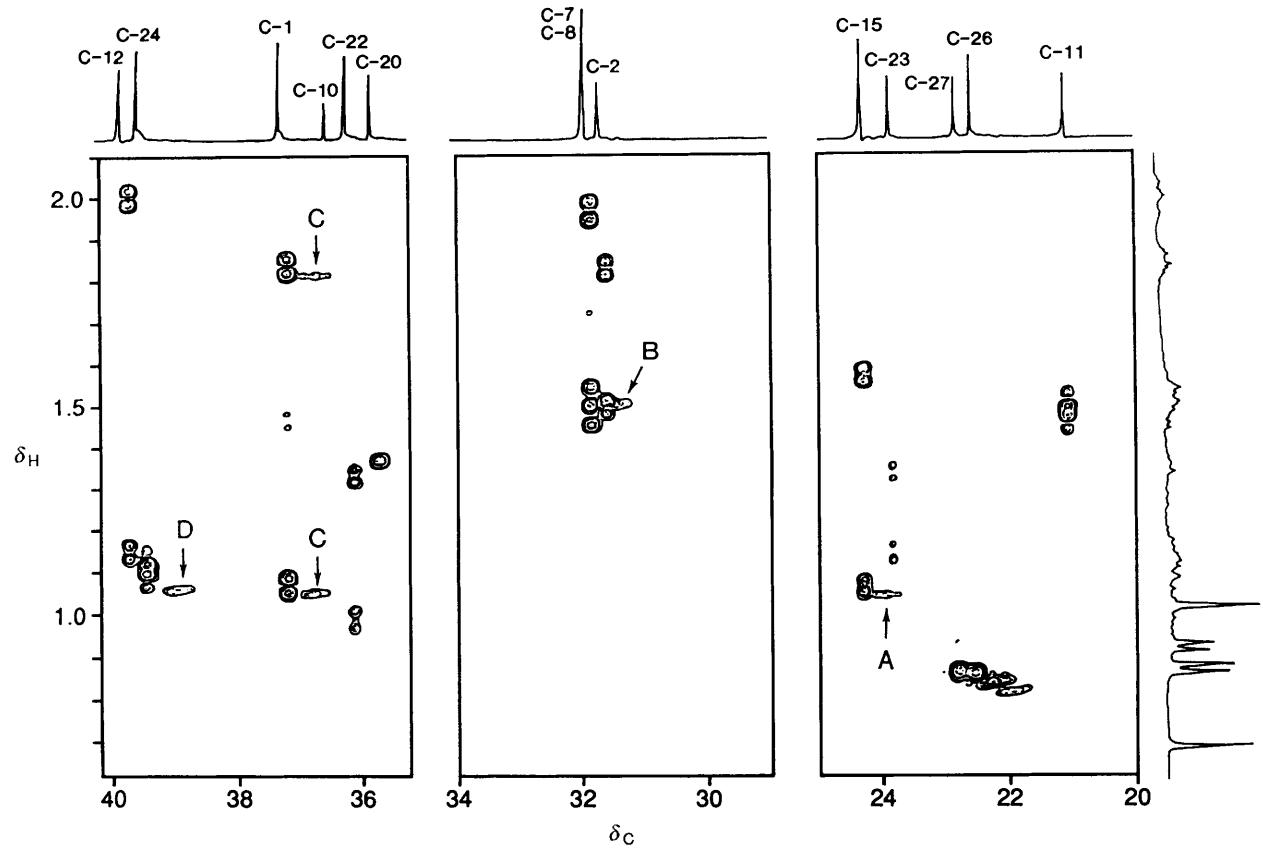


Fig. 1 Regions of the proton-decoupled ^{13}C–^{1}H COSY spectrum of [$^{13}C^{2}H$]cholesterol **6** biosynthesized from [2-$^{13}C^{2}H_3$]acetate in isolated dog hepatocytes determined at 100 MHz for ^{13}C and at 400 MHz for ^{1}H: cross peaks of (A) C-15 and 15β-^{1}H with 15α-^{2}H, (B) C-7 and 7α-^{1}H with 7β-^{2}H, (C) C-1 and 1-^{1}H with 1α- and 1β-^{2}H, and (D) C-24 and 24-*pro*-*S*-^{1}H with 24-*pro*-*R*-^{2}H

a deuterium isotope shifted signal ($^{1}\Delta\delta_C$ −0.49 and $^{2}\Delta\delta_H$ −0.023) (D). Therefore the former and the latter signals should be assigned to be the 24-pro-*S* and 24-pro-*R* hydrogens, respectively. Both cross peaks between C-1 (δ_C 37.27) and 1α-H (δ_H 1.072) and 1β-H (δ_H 1.842) of **6**, which were assigned based on the NOESY spectrum, had deuterium isotope shifted signals (C) ($^{1}\Delta\delta_C$ −0.37 and −0.43, $^{2}\Delta\delta_H$ −0.022 and −0.023). These observation suggest non-stereospecific loss of a deuteron originating from C-2 of MVA. The deuteron could be exchanged with water in the medium during metabolism of acetate to MVA, or in the equilibrium between isopentenyl pyrophosphate and γ,γ-dimethylallyl pyrophosphate. [$^{13}C^{2}H$]-Labelled lanosterol was not obtained in this experiment.

Incorporation of [1,2-$^{13}C_2$]- *and* [2-$^{13}C^{2}H_3$]-*Acetate into Lanosterol.*—We have previously reported that a fungicide, SSF-109,* caused accumulation of several 14α-methylsterols in *Botrytis cinerea*,[22] while in isolated rat hepatocytes, there was accumulation of only two 14α-methylsterols; lanosterol and 24(25)-dihydrolanosterol.[5] In order to examine the incorporation pattern of the carbon atoms of acetate into lanosterol, isolated dog hepatocytes were incubated with [1,2-$^{13}C_2$]acetate diluted with twice the amount of non-labelled acetate in the presence of SSF-109. After 3 h incubation, HPLC analysis of nonsaponifiable lipids showed accumulation of lanosterol (Fig. 3 in Experimental section). [^{13}C]-Labelled lanosterol **2** and cholesterol were isolated by preparative HPLC.

As shown in Table 3, the ^{13}C-{^{1}H} NMR spectrum of lanosterol **2** showed 10 carbons as labelled singlets (C-1, C-7, C-8, C-14, C-15, C-18, C-22, C-26, C-30 and C-32) and 10 carbon pairs as labelled doublets (C-2–C-3, C-4–C-31, C-5–C-6, C-9–C-11, C-10–C-19, C-12–C-13, C-16–C-17, C-20–C-21, C-23–C-24 and C-25–C-27). The two pairs of labelled singlet carbons of C-18 and C-14 and of C-32 and C-8 indicate 1,2-methyl migrations (C-18 from C-14 to C-13 and C-32 from C-8 to C-14) in the course of cyclization of 2,3-oxidosqualene to lanosterol **2** *via* cationic intermediate **1** as shown in Scheme 1.

Simultaneously isolated cholesterol did not show incorporation of [^{13}C]acetate as expected from the previous findings with rat hepatocytes.[5] This indicates that SSF-109 inhibited cholesterol biosynthesis almost completely at the 14α-methyl demethylation step in dog hepatocytes and caused lanosterol accumulation.

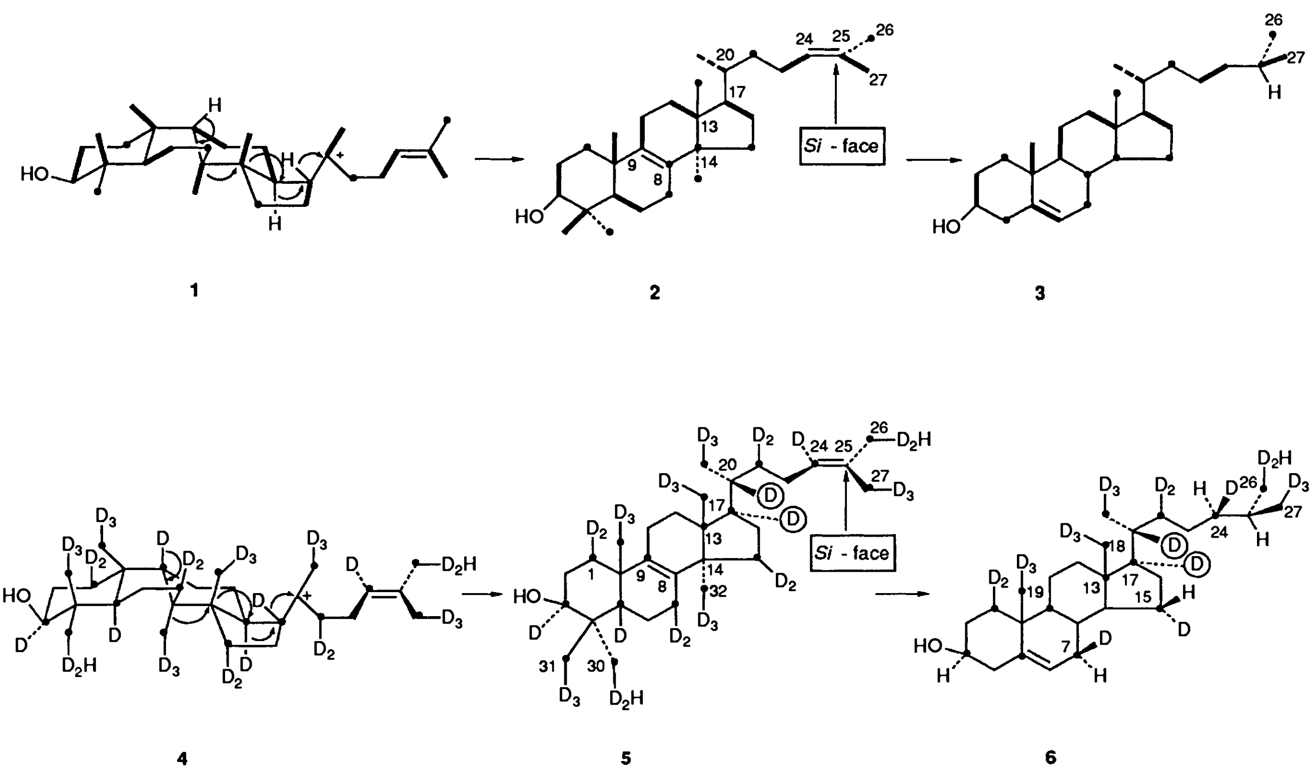
We also examined the distribution of hydrogen atoms originating from acetate by incubating [2-$^{13}C^{2}H_3$]acetate with isolated dog hepatocytes in the presence of SSF-109. [$^{13}C^{2}H$]-Labelled lanosterol **5** was obtained together with cholesterol, though the cholesterol was unlabelled. The [$^{13}C^{2}H$]-labelling patterns of **5** determined by ^{13}C-{^{1}H}{^{2}H}NMR spectroscopy are shown in Fig. 2a and in Table 3. Six methyl groups (C-18, C-19, C-21, C-27, C-31 and C-32) had the [$^{13}C^{2}H_3$]-label indicating that these carbons are originated from C-6 of MVA. In the case of higher plants, phytosterols are formed *via* cycloartenol, and C-19 of 24β-ethyl-25-dehydrolophenol[23] was demonstrated to have the [$^{13}C^{2}H_2{}^{1}H$]-label from [2-$^{13}C^{2}H_3$]-acetate. In dog hepatocytes, the [$^{13}C^{2}H_3$]-label was observed at C-19 of cholesterol **6** and lanosterol **5** indicating that they were not formed *via* a 9(19)cyclic intermediate. Four methylene groups (C-1, C-7, C-15 and C-22) and two methyl groups (C-26 and C-30) of **5** are labelled as [$^{13}C^{2}H_2$] and [$^{13}C^{2}H_2{}^{1}H$], respectively, which are originated from C-2 of MVA, and non-

* (±)-*cis*-1-(4-Chlorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)cycloheptanol.

Table 3 ^{13}C NMR spectroscopic data of biosynthesized lanosterol **2** from [1,2-$^{13}C_2$]acetate, **5** from [2-$^{13}C^2H_3$]acetate, and **9** from [1-^{13}C]acetate and [1-$^2H_2$]ethanol in isolated dog hepatocytes

		2			**5**				**9**			
Carbon	δ_C	s/d [a]	J_{CC}	(J_{CC}) [b]	$^{13}C^2Hn$	$^1\Delta\delta_{C^2H_1}$	$^1\Delta\delta_{C^2H_2}$	$^1\Delta\delta_{C^2H_3}$	$^{13}C^2Hn$	$^1\Delta\delta_{C^2H_1}$	$^1\Delta\delta_{C^2H_1}$	$^1\Delta\delta_{C^2H_2}$
C-1	35.61	s		(34)	$^{13}C^2H_2$	−0.42	−0.80		—			
C-2	27.87	d	37	(34)	—				$^{13}C^2H_2$	−0.36	−0.40	n.d. [c]
C-3	78.99	d	37	(37)	$^{13}C^2H_1$	−0.52			—			
C-4	38.90	d	36	(35)	—				^{13}C			
C-5	50.41	d	34	(34)	$^{13}C^2H_1$	−0.57			—			
C-6	18.34	d	34	(34)	—				$^{13}C^2H_2$	−0.35	−0.35	n.d.
C-7	26.52	s		(39)	$^{13}C^2H_2$	−0.41	−0.80		—			
C-8	134.41	s		*g*					^{13}C			
C-9	134.41	d	42	(42)	^{13}C				—			
C-10	37.03	d	35	(35)	—				^{13}C			
C-11	21.02	d	42	(34)	—				$^{13}C^2H_2$	−0.35 [d]	−0.35 [d]	n.d.
C-12	31.01	d	35	(34)	—				$^{13}C^2H_2$	−0.39 [e]	−0.39 [e]	n.d.
C-13	44.50	d	35	(35)	$^{13}C(\beta$-$^2H)$	(−0.08) [f]			—			
C-14	49.81	s		(33)	—				^{13}C			
C-15	30.86	s		(33)	$^{13}C^2H_2$	−0.39	−0.73		—			
C-16	28.21	d	34	(33)	—				$^{13}C^2H_2$	−0.35	−0.39	n.d.
C-17	50.41	d	34	(34)	$^{13}C(\beta$-$^2H)$	(−0.11) [f]			—			
C-18	15.75	s		(35)	$^{13}C^2H_3$	*g*	*g*	*g*				
C-19	19.16	d	35		$^{13}C^2H_3$	−0.29	−0.57	−0.89	—			
C-20	36.27	d	35	(35)	—				^{13}C			
C-21	18.65	d	35		$^{13}C^2H_3$	−0.30	−0.61	−0.91	—			
C-22	36.37	s		(35)	$^{13}C^2H_2$	−0.42	−0.78		—			
C-23	24.93	d	44	(35)	—				$^{13}C^2H_2$	−0.33	−0.37	n.d.
C-24	125.24	d	44	(74)	$^{13}C^2H_1$	−0.37			—			
C-25	130.87	d	42	(74, 42)	—				^{13}C			
C-26	25.72	s		(42)	$^{13}C^2H_2{}^1H_1$	−0.28	−0.59		—			
C-27	17.64	d	42		$^{13}C^2H_3$	−0.26	−0.54	−0.84	—			
C-30	27.97	s		(37)	$^{13}C^2H_2{}^1H_1$	−0.31	−0.62		—			
C-31	15.42	d	36		$^{13}C^2H_3$	−0.27	−0.56	−0.85	—			
C-32	24.26	s		(35)	$^{13}C^2H_3$	−0.30	−0.60	−0.90	—			

[a] s: Labelled singlet and d: labelled doublet. [b] Interunit coupling constants (Hz). [c] Not detected. [d] A β-deuterium isotope signal ($^2\Delta\delta_C{}^2{}_H$ −0.10) and a doublet (J_{CC} 33 Hz) were observed. [e] A β-deuterium isotope shifted signal ($^2\Delta\delta_{C^2H}$ −0.09) and a doublet (J_{CC} 33 Hz) were observed. [f] β-Deuterium isotope shift. [g] Not determined because of signal overlapping.



Scheme 1

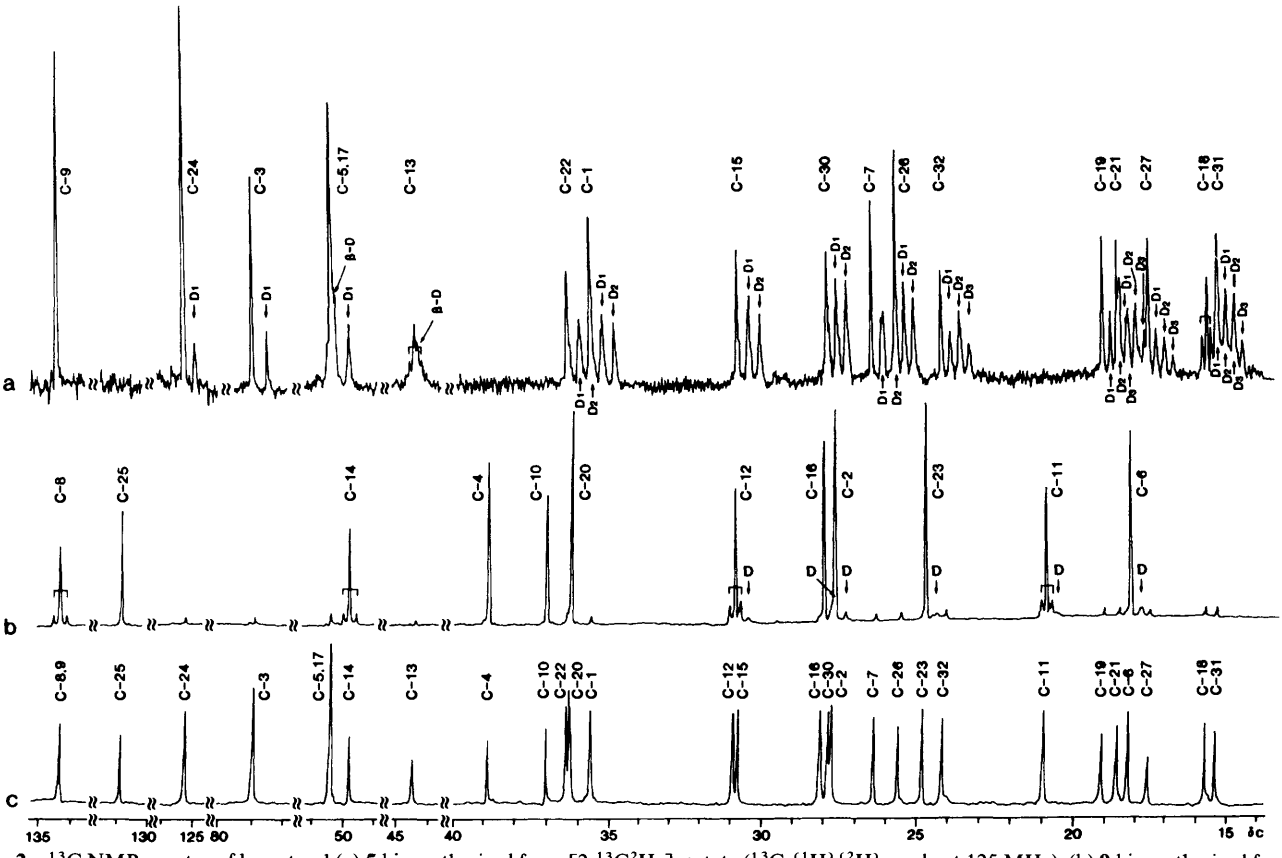
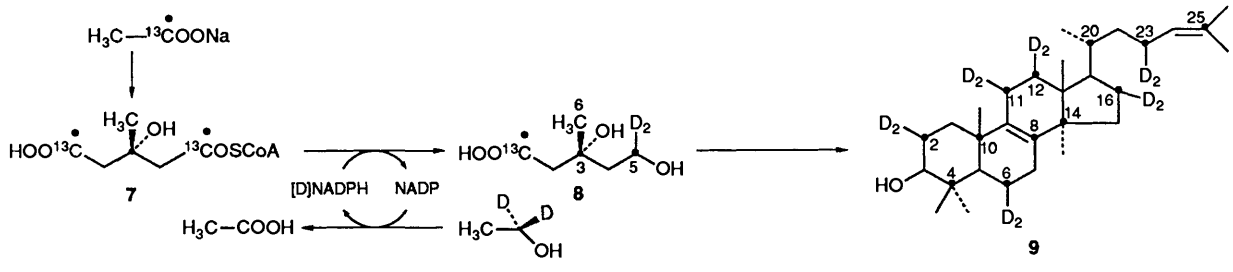


Fig. 2 ^{13}C NMR spectra of lanosterol (a) **5** biosynthesized from [2-$^{13}C^{2}H_3$]acetate (^{13}C-{^{1}H}{^{2}H} mode at 125 MHz), (b) **9** biosynthesized from [1-^{13}C]acetate and [1-$^{2}H_2$]ethanol (^{13}C-{^{1}H}{^{2}H} mode at 100 MHz) and (c) non-labelled specimen (^{13}C-{^{1}H} mode at 100 MHz)



Scheme 2

stereospecific deuterium exchange was observed at C-1, C-7 and C-15 in the proton-decoupled ^{13}C-^{1}H COSY spectrum. Three carbons bearing one deuterium atom (C-3, C-5 and C-24) are originated from C-4 of MVA. The β-deuterium isotope shifted signals, which were observed at C-13 ($^{2}\Delta\delta_C$ −0.08) and C-17 ($^{2}\Delta\delta_C$ −0.11) in the ^{13}C-{^{1}H}NMR spectrum, confirmed the 1,2-deuterium migrations from C-13 to C-17 and from C-17 to C-20 as observed in cholesterol **6**. C-9 was observed as [^{13}C] and is consistent with the loss of a deuterium atom to terminate the backbone rearrangement.

Isolated dog hepatocytes were found to have very high metabolic activity to transfer the endogenous acetate into sterols. Even when [1,2-$^{13}C_2$]acetate was diluted with twice the amount of non-labelled acetate, interunit ^{13}C–^{13}C coupling was observed in **2**: coupling constants are shown in parentheses in Table 3. The signals, C-13 and C-17 of **5**, were complicated due to interunit couplings between C-13 and C-18 and/or C-17 with deuterium isotope shifts. Doublet signals (J_{CC} 35 Hz) observed at C-13 and C-18 in **5** agreed with the methyl migration from C-14 to C-13. Carbons (C-2, C-4, C-6, C-8, C-10, C-11, C-12, C-14, C-16, C-20, C-23 and C-25) of **5** were virtually not observed, because they are derived from C-1 of acetate and thus from C-3 and C-5 of MVA. These results suggest a very small pool size of intermediates in the metabolic pathway of acetate to lanosterol in dog hepatocytes which differed from that of yeast and plant systems.[4]

Incorporation of [1-^{13}C]*Acetate and* [1-$^{2}H_2$]*Ethanol into Lanosterol* **9**.—When 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) **7** is reduced to MVA **8** by HMG-CoA reductase, the hydrogen atoms at C-5 of MVA are introduced from the 4-pro-*R* hydrogen of NADPH.[26] The hydrogen atom of NADPH is transferred from ethanol when ethanol is oxidized to acetic acid[26] as shown in Scheme 2. To confirm the distribution of C-1 of acetate and the incorporation patterns of the hydrogens at C-5 of MVA into lanosterol, [1-^{13}C]acetate and [1-$^{2}H_2$]ethanol were incubated simultaneously with isolated dog hepatocytes in the presence of SSF-109. HPLC analysis of non-saponifiable lipids showed accumulation of lanosterol **9** and a small amount of dihydrolanosterol. The [^{13}C,^{2}H]-labelling patterns of lanosterol **9** were determined by ^{13}C-{^{1}H}-{^{2}H}NMR spectroscopy and are shown in Fig. 2b and Table 3. Twelve carbons (C-2, C-4, C-6, C-8, C-10, C-11, C-12, C-14, C-16, C-20, C-23 and C-25) were labelled. Two pairs of carbons, C-

8–C-14 (J_{CC} 42 Hz) and C-11–C-12 (J_{CC} 33 Hz), were observed as doublets due to interunit coupling. C-11 and C-12 also had a β-deuterium shifted signal ($^2\Delta\delta_C$ −0.10 and −0.09, respectively). [^{13}C,^{2}H]-Labelled signals were observed at six carbons (C-2, C-6, C-11, C-12, C-16 and C-23) with α-deuterium isotope shifts ($^1\Delta\delta_C$ −0.33 to −0.40) in agreement with the origin of these carbons being from C-5 of MVA **8**. Dilution of the generated [^{2}H]NADPH with endogenous NADPH might interfere with the determination of the second deuterium atom incorporated simultaneously. We confirmed the deuterium atoms distributed at both axial-($^1\Delta\delta_C$ −0.35 to −0.40) and equatorial-($^1\Delta\delta_C$ −0.35 to −0.36) positions of C-2, C-6 and C-16 by the proton-decoupled ^{13}C–^{1}H COSY spectrum. As two protons at C-11 and also at C-12 have almost the same chemical shift, axial and equatorial distributions of deuterium atoms on these carbons were not able to be determined. One of the hydrogen atoms at C-11 and C-12 should be replaced by a hydrogen from NADPH when farnesyl pyrophosphate was converted into squalene *via* presqualene[24,25] but the intensity of the α-deuterium shifted signals at C-11 and C-12 was almost the same as those of other labelled carbons. A sufficient amount of [1-$^2H_2$]ethanol might supply [^{2}H]NADPH until the presqualene was transformed to squalene. Cholesterol incorporated neither [^{13}C] nor [^{2}H].

Conclusions

The distributions of the carbon and hydrogen atoms of acetic acid in the biosynthesis of lanosterol and cholesterol were examined by incubating [1,2-$^{13}C_2$]- and [2-$^{13}C^2H_3$]-acetate with isolated dog hepatocytes either in the presence or in the absence of a 14α-methyl demethylation inhibitor, SSF-109. The methyl migrations and hydride shifts originally proposed by Ruzicka *et al.* in 'the biogenetic isoprene rule'[27] were confirmed by ^{13}C NMR spectroscopy in the biosynthesis of lanosterol and also in cholesterol. In the conversion of lanosterol into cholesterol with extrusion of three methyl groups and a double bond migration, two hydrogen atoms at C-7 and C-15 were replaced stereospecifically. The proton-decoupled ^{13}C–^{1}H COSY spectrum showed that introduction of hydrogen atoms at these carbons took place from the 7α- and 15β-sides and the reduction of the 24(25) double bond occurred stereospecifically from the *Si*-face at C-25 of lanosterol. The distribution of hydrogens originating from C-5 of MVA was examined by a co-incubation of [1-^{13}C]acetate and [1-$^2H_2$]ethanol with dog hepatocytes, which revealed that the hydrogens at C-2, C-6, C-11, C-12, C-16 and C-23, were transferred from ethanol *via* NADPH.

Experimental

NMR spectra were obtained in [^{2}H]-chloroform using tetramethylsilane as an internal standard (δ_C 0 and δ_H 0). ^{13}C-{^{1}H} NMR spectra were recorded on a Varian XL-400 instrument at 100.577 MHz. Typical F.T. NMR conditions were: spectral width (s.w.), 16 501.7 Hz; acquisition time (a.t.), 0.909 s; and pulse flip angle, 10°. ^{13}C-{^{1}H}{^{2}H} NMR spectra were determined on a JEOL GSX-500 instrument at 125.77 MHz under the following conditions: s.w., 10 330.6 Hz; a.t., 0.793 s; pulse delay time (p.d.), 2.0 s; and pulse width (p.w.), 6.0 μs. Proton-decoupled ^{13}C–^{1}H COSY, ROESY and NOESY spectra were obtained from a Varian XL-400 instrument at 100.577 and 399.941 MHz for ^{13}C and ^{1}H NMR, respectively, under the following conditions: s.w., 7837.0 Hz, a.t., 0.131 s; p.d., 0.869 s; number of fid's (n.i.), 256; and data size (zero filling to), 4096 × 2048 for proton-decoupled ^{13}C–^{1}H COSY, s.w., 2469.7 Hz; a.t., 0.207 s; p.d., 0.700 s; n.i., 128; and data size (zero filling to), 2048 × 2048 for ROESY, and s.w., 1291.8 Hz, a.t., 0.198s; p.d., 1.000 s; n.i., 128; and data size (zero filling to), 1024 × 1024 for NOESY. ^{1}H NMR spectra were obtained from a Varian VXR-200 instrument at 200.057 MHz using the following conditions: s.w., 3200 Hz; a.t., 2.9 s; p.d., 2.0 s; and p.w., 12 μs. The maximum entropy Fourier spectral deconvolution was processed by New Methods Research inc. (NMRi) system on DEC VAX 3200. *J* Values are given in Hz.

HPLC was performed with a Waters 600 multisolvent delivery system equipped with a Shimadzu SPD-6A UV detector at λ 210 nm. For analysis, a Develosil ODS T-5 column (150 × 4.6 mm i.d.) (Nomura Chemical, Aichi, Japan) connected with a YMC-pack ODS A312 (S-5) column (150 × 6 mm i.d.) (Yamamura Chemical Lab., Kyoto, Japan) were eluted with methanol (1 cm^3 min^{-1}). For preparative purpose, a Develosil ODS T-7 (S-7) column (250 × 20 mm i.d.) connected with a YMC-pack SH343 (S-15) ODS column (250 × 20 mm i.d.) was eluted with methanol (9 cm^3 min^{-1}). Sodium [2-$^{13}C^2H_3$]acetate was purchased from Cambridge Isotope Laboratories (UK), and sodium [1,2-$^{13}C_2$]acetate from Amersham Lab. (UK), and sodium [1-^{13}C]acetate, and [1-$^2H_2$]ethanol from Aldrich (Milwaukee, USA). Isotope enrichment were 99 atom % for ^{13}C and 98 atom % for ^{2}H.

Preparation and Incubation of Isolated Dog or Rat Hepatocytes.—Parenchymal cells were isolated from a male or a female beagle dog (EDM) of 8 to 11 months old or from male Wistar rats, weighing 300 g. The liver was perfused with 0.08% collagenase according to Moldéus *et al.*[28] as described previously.[29] The cells obtained were suspended in a rotary round bottom flask at a density of 1.2 × 10^7 cells cm^{-3} in Krebs–Henselit buffer supplemented with bovine serum albumin (BSA) (0.2%) and preincubated at 37 °C for 15 min under an atmosphere of 5% CO_2–95% O_2 with or without SSF-109. Isotope labelled compound was then added to the suspension and incubation was continued for a total of 3 h.

Incorporation of [1-^{14}C]*Acetate and* [2-^{14}C]*Mevalonate into Cholesterol in Rat or Dog Hepatocytes.*—Hepatocytes (2 × 10^7 cells/0.9 cm^3 tube^{-1}) isolated from a rat (male Wistar, 300 g) or a dog liver were precincubated for 15 min at 37 °C. Sodium [1-^{14}C]acetate (1 × 10^{-3} mol dm^{-3}, 0.15 MBq/tube) or sodium [2-^{14}C]mevalonate (0.25 × 10^{-3} mol dm^{-3}, 15 KBq/tube) was incubated for 60 min. Methanol (1.5 cm^3) and KOH (10 mol dm^{-3}; 0.3 cm^3) were added to the incubation mixture which was then incubated for 60 min at 70 °C. Non-saponifiable lipids, extracted by the Bligh and Deyer method,[30] were subjected to TLC (Merck, Art. 13794) and developed with hexane–chloroform–ethyl acetate (4:1:1). The cholesterol region was made visible with iodine and then scraped into a vial. Radioactivity was determined in Scintisol by a liquid scintillation counter.

Isolation of [^{13}C]*Cholesterol* **3** *Biosynthesized from* [1,2-$^{13}C_2$]*Acetate.*—Suspensions of isolated dog hepatocytes (160 cm^3) were preincubated for 15 min at 37 °C and then a mixture of sodium [1,2-$^{13}C_2$]acetate (81 mg) and nonlabelled sodium acetate (158 mg) dissolved in water (3 cm^3) was added every 40 min in three portions (final 18 × 10^{-3} mol dm^{-3}). The suspension was incubated for 3 h at 37 °C. Potassium hydroxide (24 g) in methanol (240 cm^3) was added and the mixture was stirred for 1 h at 70 °C. After addition of water (80 cm^3), non-saponifiable lipids were extracted with hexane (3 × 250 cm^3) and then dichloromethane (300 cm^3), followed by washing with a mixture of methanol–water (1:1, 3 × 100 cm^3). The non-saponifiable lipids (50.1 mg) were subjected to preparative HPLC which gave [^{13}C]cholesterol **3** (12 mg); δ_H 0.677 (3 H, s, 18-H), 0.862 (3 H, d, *J* 6.6, 26-H), 0.867 (3 H, d, *J* 6.6, 27-H), 0.913 (3 H, d, *J* 6.4, 21-H), 1.008 (3 H, s, 19-H), 3.523 (1 H, m, 3-H) and 5.352 (1 H, d, *J* 5.4, 6-H).

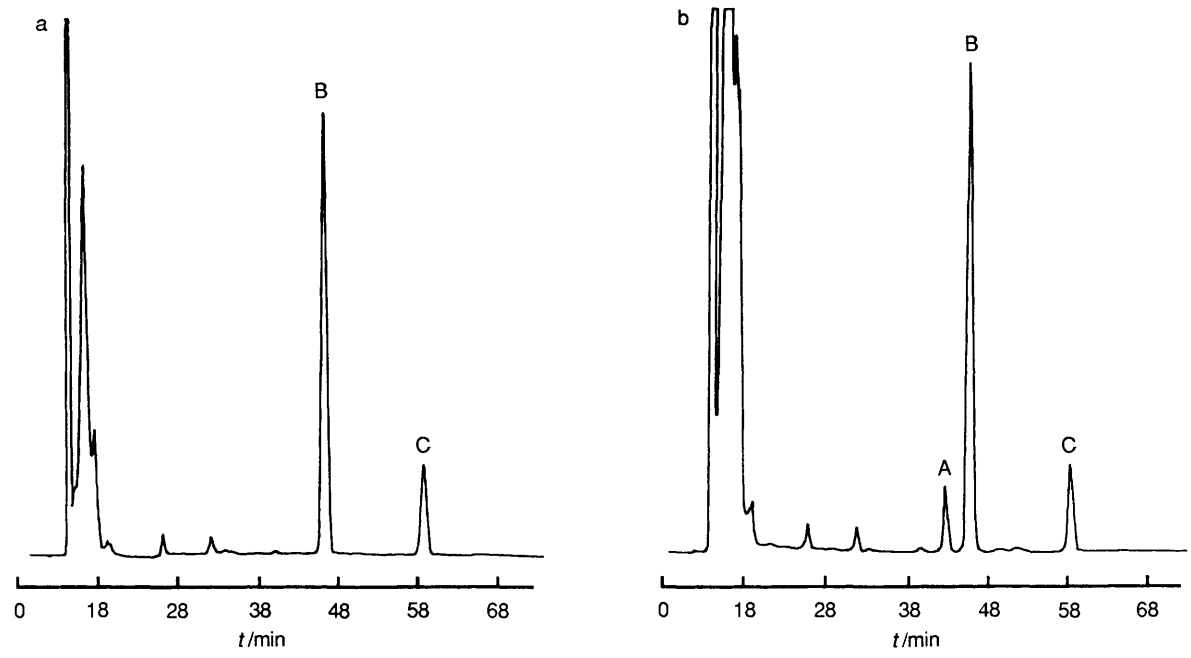


Fig. 3 HPLC profile of non-saponifiable lipid fraction obtained from incubation of isolated dog hepatocytes and [1,2-$^{13}C_2$]acetate in the absence (a) or presence (b) of SSF-109. A; lanosterol, B; cholesterol and C; squalene. HPLC conditions were; column, a Develosil ODS T-5 connected with a YMC-pack A312; solvent, methanol 1 cm^3 min^{-1}, detection at λ 210 nm.

Isolation of [$^{13}C^2H$]*Cholesterol* **6** *Biosynthesized from* [2-$^{13}C^2H_3$]*Acetate.*—A suspension of isolated dog hepatocytes (260 cm^3) was preincubated for 15 min at 37 °C, and then non-diluted sodium [2-$^{13}C^2H_3$]acetate (64 mg) in water (1 cm^3) was added. After incubation for 1 h, sodium [2-$^{13}C^2H_3$]acetate (64 mg) was added again (final 6 × 10^{-3} mol dm^{-3}) and incubation was continued for 2 h. Cholesterol was isolated from the non-saponifiable lipids by preparative HPLC as described above and crystallized from methanol to give [$^{13}C^2H$]cholesterol **6** (22 mg).

Isolation of [^{13}C]*Lanosterol* **2** *Biosynthesized from* [1,2-$^{13}C_2$]*Acetate.*—Isolated dog hepatocytes (400 cm^3) were preincubated with SSF-109 (80 mg dissolved in dimethyl sulphoxide, 1 cm^3) for 15 min at 37 °C. A mixture of sodium [1,2-$^{13}C_2$]acetate (202 mg) and nonlabelled sodium acetate (394 mg) dissolved in water (3 cm^3) was added to the solution every 40 min in three portions (final 18 × 10^{-3} mol dm^{-3}) and the suspension was further incubated for 2 h at 37 °C. A solution of KOH (60 g) in methanol (600 cm^3) was added to the incubation mixture and stirred for 1 h at 70 °C. After the addition of water (200 cm^3), non-saponifiable lipids were extracted with hexane (3 × 600 cm^3) and then dichloromethane (600 cm^3) and washed with a mixture of methanol–water (1:1, 3 × 200 cm^3). Solvent evaporation gave a residue (321 mg), which was subjected to preparative HPLC to afford [^{13}C]lanosterol **2** (0.7 mg) and nonlabelled cholesterol (32.6 mg); ^{1}H NMR spectrum for **2**; δ_H 0.689 (3 H, s, 18-H), 0.812 (3 H, s, 31-H), 0.876 (3 H, s, 32-H), 0.912 (3 H, d, *J* 6.0, 21-H), 0.980 (3 H, s, 19-H), 1.001 (3 H, s, 30-H), 1.603 (3 H, s, 27-H), 1.683 (3 H, s, 26-H), 3.236 (1 H, dd, *J* 11.0 and 5.0, 3-H) and 5.097 (1 H, m, 24-H). HPLC analysis of the residue is shown in Fig. 3.

Isolation of [$^{13}C^2H$]*Lanosterol* **5** *Biosynthesized from* [2-^{13}C-$^2H_3$]*Acetate.*—A suspension of isolated dog hepatocytes (520 cm^3) was preincubated with SSF-109 (104 mg dissolved in dimethyl sulphoxide, 1 cm^3) for 15 min at 37 °C. A mixture of sodium [2-$^{13}C^2H_3$]acetate (256 mg) and nonlabelled sodium acetate (512 mg) in water (3 cm^3) was added in three portions (every 40 min, final 18 × 10^{-3} mol dm^{-3}), and incubation was continued. After 3 h of incubation, non-saponifiable lipids (201 mg) were obtained as described above. [$^{13}C^2H$]Lanosterol **5** (0.9 mg) and nonlabelled cholesterol (31.9 mg) were isolated by preparative HPLC.

Isolation of [^{13}C,^2H]*Lanosterol* **9** *Biosynthesized from* [1-^{13}C]*Acetate and* [1-$^2H_2$]*Ethanol.*—Suspension of isolated dog hepatocytes (669 cm^3) were preincubated with SSF-109 (134 mg dissolved in 1 cm^3 dimethyl sulphoxide) for 15 min at 37 °C. A mixture of sodium [1-^{13}C]acetate (333 mg) and nonlabelled sodium acetate (658 mg) in water (3 cm^3) and [1-$^2H_2$]ethanol (2 g) in water (38 cm^3) were then added to the suspension in three portions (every 40 min, final 18 × 10^{-3} mol dm^{-3} for acetate and 43 × 10^{-3} mol dm^{-3} for ethanol). After 3 h of total incubation, non-saponifiable lipids (551 mg) were obtained. [^{13}C,^2H]Lanosterol **9** (4.5 mg) and [^{13}C,^2H]dihydrolanosterol (1.2 mg) were isolated by preparative HPLC. Non-labelled cholesterol (71 mg) was also isolated. ^{1}H NMR spectrum for dihydrolanosterol; δ_H 0.688 (3 H, s, 18-H), 0.811 (3 H, s, 31-H), 0.864 (3 H, d, *J* 6.6, 26-H), 0.869 (3 H, d, *J* 6.6, 27-H), 0.880 (3 H, s, 32-H), 0.889 (3 H, d, *J* 6.0, 21-H), 0.983 (3 H, s, 19-H), 1.001 (3 H, s, 30-H) and 3.24 (1 H, m, 3-H).

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