Migration of Tritium to C-24 during the Metabolism of 24-Methylene[23,23,25-$^3H_3$]cholesterol by the Insect *Tenebrio molitor*

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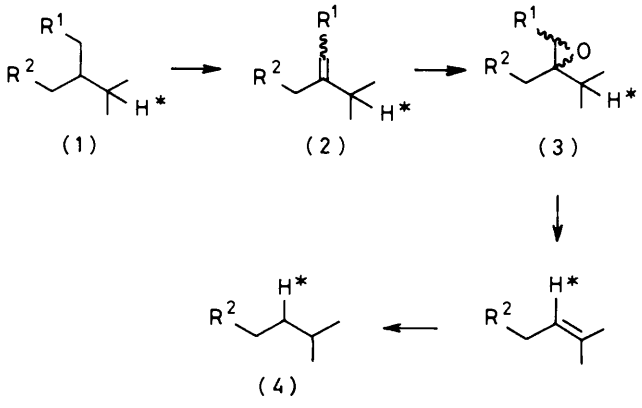
24-Methylene[23,23,25-$^3H_3$]cholesterol was transformed by *Tenebrio molitor* larvae into [23,23,24-$^3H_3$]cholesterol; the tritium label distribution in the cholesterol isolated from the larvae compared with that in the administered precursor is in agreement with the migration of hydrogen from C-25 to C-24 during the dealkylation process.

Many phytophagous insects need cholesterol for their growth and development. They obtain it through the dealkylation of C_{28} and C_{29} phytosterols, a process which involves the removal of the alkyl group at C-24.[1] Scheme 1 reports the dealkylation process leading from a typical C_{29} phytosterol, such as sitosterol (1a), to cholesterol (4).[2] A curious feature of the above process is the migration of the 25 hydrogen atom to the C-24 position rather than its loss during the metabolic sequence (Scheme 1).[3]

While the mechanism of the C_{29} phytosterol metabolism is known, the metabolism of C_{28} phytosterols has been less studied, so that many questions are still unanswered. One important point is the mechanism of the removal of the methyl group at C-24: it could resemble other well known demethylation processes, *e.g.* the removal of C-19 in oestrogen biosynthesis or the demethylations at C-4 or C-14 in the biosynthesis of cholesterol from lanosterol;[4] alternatively, it could be analogous to the de-ethylation process in insects. In the former case the enzymic systems by which the dealkylation occurs are different for C_{28} and C_{29} phytosterols, whereas in the latter case both ethyl and methyl groups could be removed by a single enzymic system with a low degree of specificity. The latter hypothesis is supported by the intervention, in the C_{28} phytosterol metabolism, of intermediates analogous to those of C_{29} phytosterol metabolism: we have recently observed[5] that 24-methylenecholesterol (2b) and the corresponding 24,28-epoxides (3b) are metabolized by *Tenebrio molitor* larvae, and Rees *et al.*[6] have shown the intermediacy of 24-methylenecholesterol epoxide (3b) during the formation of cholesterol in *Schistocerca* mid-gut microsomes. Further light would be cast on the mechanism of the removal of the methyl group at C-24 if it were known whether the hydrogen migration from C-25 to C-24, which is typical of C-29 phytosterol dealkylation, occurs also in the C-28 phytosterol dealkylation†. Since we have observed that 24-methylenecholesterol (2b) is metabolized by *Tenebrio molitor* larvae, we chose to utilize this phytosterol, labelled at C-23 and C-25, to verify the presence of a C-24 tritium atom in the cholesterol produced during the metabolism.



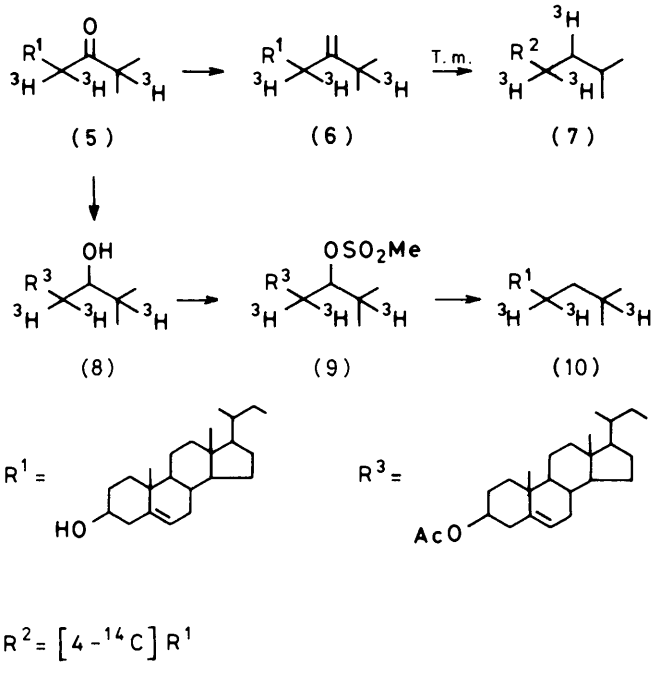
Scheme 1.

Results and Discussion

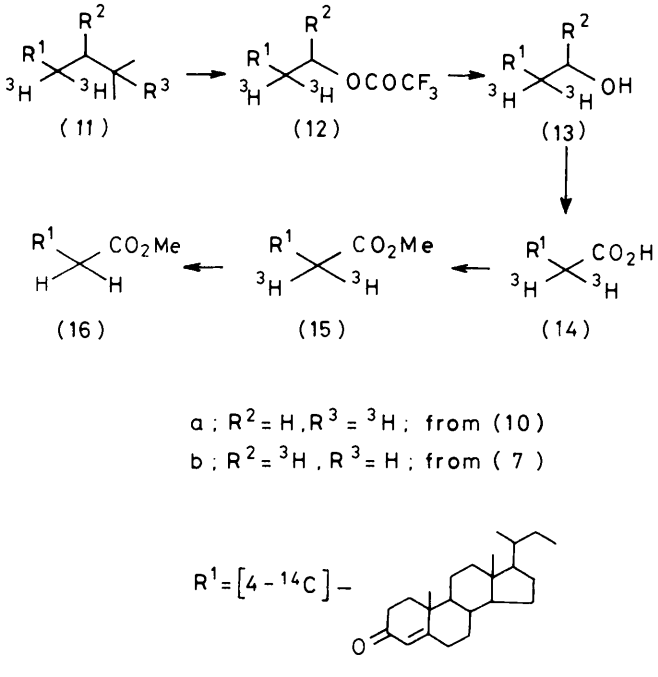
Our plan was to transform 24-methylene[23,23,25-$^3H_3$]cholesterol (6), synthesized[5] from 24-oxo[23,23,25-$^3H_3$]cholesterol (5),[8] into labelled cholesterol (7) by administration to *Tenebrio molitor* larvae (Scheme 2); chemical degradation of the obtained cholesterol, according to the sequence shown in Scheme 3, would indicate whether the sample contained tritium at C-24.

Before starting with the biological experiment we have to determine the tritium label distribution in the precursor (6); we had also to check the degradation process of Scheme 3 in order to verify that it was suitable for our purpose since it is based on a reaction, oxidation with trifluoroperacetic acid,[9] the mechanism of which has not been demonstrated. To this aim we transformed 24-oxo[23,23,25-$^3H_3$]cholesterol (5), from which (6) had been synthesized, into cholesterol (10) through a sequence which did not change the tritium label distribution (Scheme 2); (5) was acetylated and submitted to $NaBH_4$ reduction to give 24-hydroxycholesteryl acetate (8), which was mesylated and reduced with $LiAlH_4$ to [23,23,25-$^3H_3$]cholesterol (10). (10) Was mixed with [4-^{14}C]cholesterol and, after Oppenauer oxidation to [23,23,25-$^3H_3$, 4-^{14}C]cholest-4-en-3-one (11a), was submitted to the chemical degradation of the side chain according to Scheme 3. Trifluoroperacetic acid oxidation[9] of (11a) removed the isopropyl terminal group of the side chain and afforded the primary reaction product, 24-trifluoroacetoxy[23,23-$^3H_2$, 4-^{14}C]chol-4-en-3-one (12a) which

† Recently Maruyama *et al.*[7] have shown that, in the metabolism of campesterol (1b) and 24-methylenecholesterol (2b) in *Bombyx mori*, the hydrogen at C-25 was not lost during the demethylation process.



Scheme 2.



Scheme 3.

was hydrolysed to the 24-hydroxy-derivative (13a). The radioactivity data reported in Table 1 show that, during this reaction, *ca.* 40% of the tritium is lost, so indicating that this amount of tritium is present at the C-25 position. Compound (13a) was oxidized with pyridinium dichromate [10] in dry dimethylformamide to 3-oxo[23,23-$^3H_2$,4-^{14}C]chol-4-en-24-oic acid (14a), which was counted as the methyl ester (15a). As shown in Table 1 no valuable loss of tritium was observed in the oxidation of the primary alcoholic group of (13a) to the carboxy-group of (14a): this fact demonstrates that: (*a*) whatever the mechanism of trifluoroperacetic acid oxidation, no migration of ^3H from C-25 to C-24 occurs during this reaction; (*b*) no exchange in the position α to the carboxy-group occurs during the PDC oxidation of (13a). Finally, the presence of the remaining label at C-23 was shown by the virtual complete loss of tritium (Table 1) when the methyl ester (15a) was submitted twice to MeOH/MeO$^-$ exchange.

Table 1. Specific activities, $^3H/^{14}C$ ratios and relative molar radioactivity (RMA) of cholesterol (10) and its chemical degradation products

Compd.	D.p.m. of ^{14}C/mmol	$^3H/^{14}C$	RMA
(10)	7.16×10^4	9.0	100
(11a)	7.20×10^4	8.9	99
(13a)	6.98×10^4	5.6	62
(15a) [a]	7.34×10^4	5.4	60
(16a) [b]	7.23×10^4	0.4	4

[a] Before MeOH/MeO$^-$ exchange. [b] After MeOH/MeO$^-$ exchange.

Table 2. Specific activities, $^3H/^{14}C$ ratios and relative molar radioactivity (RMA) of cholesterol (7) and its chemical degradation products

Compd.	D.p.m. of ^{14}C/mmol	$^3H/^{14}C$	RMA
(7)	1.13×10^5	13.6	100
(11b)	1.14×10^5	13.7	101
(13b)	1.15×10^5	13.2	97
(15b) [a]	1.08×10^5	8.2	60
(16b) [b]	1.10×10^5	0.9	6

[a] Before MeOH/MeO$^-$ exchange. [b] After MeOH/MeO$^-$ exchange.

The above results indicate the suitability of our approach, so that we could start with the biological experiment. 24-Methylene[23,23,25-$^3H_3$]cholesterol (6) was fed, together with [4-^{14}C]sitosterol as internal standard, to young *Tenebrio molitor* larvae; after 4 days the larvae were sacrificed, macerated in ethanol, and hydrolysed; the sterol fraction was benzoylated and submitted to preparative argentation t.l.c. from which a mixture of sitosteryl and cholesteryl benzoates was obtained. Preparative h.p.l.c. of this mixture afforded pure cholesteryl benzoate, which was submitted to alkaline hydrolysis to give [23,23,24-$^3H_3$, 4-^{14}C]cholesterol (7). Oppenauer oxidation of (7) gave (11b) which was submitted to the chemical degradation of the side-chain illustrated above for (11a). The intermediates of the sequence were crystallized to constant specific activities and $^3H/^{14}C$ ratios; the obtained values, reported in Table 2, show that no valuable loss of tritium occurred during the trifluoroperacetic acid oxidation, indicating that no tritium was present in the C-25 position; however, *ca.* 40% of tritium was lost during the PDC oxidation: this amount was therefore located at C-24. The remaining tritium was present at C-23 as indicated by its almost complete loss during MeOH/MeO$^-$ exchange.

The above results fully agree with the migration of the C-25 hydrogen of 24-methylenecholesterol to C-24 of cholesterol during the metabolism of the former compound in *Tenebrio molitor*; in this respect, the dealkylation processes of the C_{28} and C_{29} phytosterols are analogous.

Experimental

All m.p.s are uncorrected. ^1H N.m.r. spectra were recorded on a Varian XL-100 or on a Brucker W-P 80 spectrometer using $SiMe_4$ as internal standard. Chromatography was carried out on silica gel S (Riedel-De Haen AG) by the flash procedure.[11]

Analytical and preparative t.l.c. were carried out on Merck 60 F_{254} silica gel plates (0.25 mm thickness) and the spots were detected under u.v. light and/or spraying with 50% aqueous sulphuric acid and heating at 110 °C for 5 min. 'Work-up' refers to dilution with water, extraction with an organic solvent, washing to neutrality, drying over Na_2SO_4, filtration, and evaporation under reduced pressure. Radioactive samples were counted on a Packard Tri-Carb 3320 liquid-scintillation counter; the samples were dissolved into 10 ml of a solution consisting of 0.65% (w/v) 2,5-diphenyloxazole and 0.013% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene–dioxan (1 : 1, v/v). G.l.c. was carried out with a Carlo-Erba Fractovap 2 400 V instrument on a 2-m column packed with 2.5% SE-30 at 250 °C. I.r. spectra were recorded on a Perkin-Elmer 681 instrument as $CHCl_3$ solutions. Mass spectra were obtained on a Varian MAT 112 instrument.

[23,23,25-$^3H_3$]*Cholesterol* (10).—24-Oxo[23,23,25-$^3H_3$]cholesterol (5) (5.20×10^6 d.p.m. of ^3H, spec. act. 2.62×10^7 d.p.m./mg), diluted to 20 mg with cold material, was acetylated overnight; the crude reaction product in 95% ethanol (25 ml) was reduced with $NaBH_4$ (25 mg) to yield 24-hydroxy-[23,23,25-$^3H_3$]cholesteryl acetate (8) (18 mg). Freshly distilled methanesulphonyl chloride (0.2 ml) was added to a cooled solution of (8) in dry pyridine (1 ml) and the mixture was set aside overnight at 0 °C; work-up gave the crude mesylation product (9) (19 mg) which was dissolved in dry ether (10 ml) and stirred for 4 h at room temperature with $LiAlH_4$ (20 mg). The excess of $LiAlH_4$ was destroyed with dilute HCl and work-up afforded 15 mg of crude product (a mixture of cholesterol plus an elimination product) from which pure [23,23,25-$^3H_3$]cholesterol (10) (8 mg, 2.10×10^6 d.p.m. of ^3H) was obtained by preparative t.l.c. on 20% $AgNO_3$–silica gel (hexane–benzene 6 : 4, two elutions) on the acetylated mixture followed by 5% ethanolic KOH hydrolysis.

[23,23,25-$^3H_3$, 4-^{14}C]*Chol-4-en-3-one* (11a).—[23,23,25-$^3H_3$]-Cholesterol (10) (8 mg) was mixed with [4-^{14}C]cholesterol (2.33×10^5 d.p.m. of ^{14}C, spec. act. 2.50×10^8 d.p.m./mg, the Radiochemical Centre, Amersham), diluted with cold material to 1.25 g and submitted to Oppenauer oxidation [9] to give (11a) (930 mg).

24-*Trifluoroacetoxy*[23,23,-$^3H_2$, 4-^{14}C]*chol-4-en-3-one* (12a). —Compound (11a) (930 mg) was oxidized according to the procedure of Manley *et al.*[9] to give, after work-up, 560 mg of crude product which, submitted to flash chromatography (hexane–ethyl acetate 80 : 20), yielded unchanged (11a) (150 mg) and (12a) (175 mg; 16%), the remainder being more polar products. Compound (12a) was crystallized from methanol, m.p. 92—93 °C; δ($CDCl_3$) 0.70 (s, 18-Me), 0.93 (d, *J* 6 Hz, 21-Me), 1.17 (s, 19-Me), 4.30 (t, *J* 6 Hz, 24-CH_2), and 5.71 (s, 4-CH); $\nu_{max.}$ ($CHCl_3$) 1 780 and 1 660 cm^{-1}; *m/z* 454 (M^+).

24-*Hydroxy*[23,23-$^3H_2$, 4-^{14}C]*chol-4-en-3-one* (13a).—Compound (12a) (175 mg) was treated with 5% ethanolic KOH (15 ml) and stirred at room temperature for 1 h. Ethanol was removed under reduced pressure to afford on work-up (13a) (120 mg, 87%) which crystallized from acetone, had m.p. 129—130 °C (lit.,[9] 131.5—133.5 °C); δ($CDCl_3$) 0.71 (s, 18-Me), 0.92 (d, *J* 6 Hz, 21-Me), 1.19 (s, 19-Me), 3.61 (t, *J* 6 Hz, 24-CH_2), and 5.72 (s, 4-CH).

3-*Oxo*[23,23-$^3H_2$, 4-^{14}C]*chol-4-en-24-oic Acid* (14a).—A solution of (13a) (120 mg) in dry *N*,*N*-dimethylformamide (1 ml) was stirred at room temperature with pyridinium dichromate (250 mg). After 8 h work-up afforded a crude product which, purified by chromatography (hexane–ethyl acetate, 70 : 30) gave (14a) (82 mg, 66%); this, crystallized from aqueous acetic acid, had m.p. 184—186 °C (lit.,[12] 185—187.5 °C); δ($CDCl_3$) 0.72 (s, 18-Me), 0.94 (d, *J* 6 Hz, 21-Me), 1.19 (s, 19-Me), and 5.74 (s, 4-CH).

Methyl 3-*Oxo*[23,23-$^3H_2$, 4-^{14}C]*chol-4-en-24-oate* (15a).—Compound (14a) (82 mg) dissolved in $CHCl_3$ (10 ml) was esterified with CH_2N_2 quantitatively to yield (15a); this, crystallized from methanol, had m.p. 126—127 °C (lit.,[12] 127—128 °C); δ($CDCl_3$) 0.72 (s, 18-Me), 0.93 (d, *J* 6 Hz, 21-Me), 1.18 (s, 19-Me), 3.67 (s, OCH_3), and 5.74 (s, 4-CH).

MeOH/MeO^- *Exchange on* (15a).—Compound (15a) (85 mg) was dissolved in 10 ml of a 0.1M-solution of MeONa in MeOH and heated at 65 °C under N_2 for 18 h. The solution was cooled and the solvent evaporated under reduced pressure; work-up afforded (16a) (68 mg), identical by t.l.c., g.l.c., and ^1H n.m.r. with (15a).

Administration of 24-*Methylene* [23,23,25-$^3H_3$]*cholesterol to* Tenebrio molitor *Larvae and isolation of* [23,23,24-$^3H_3$]-*Cholesterol* (7).—A mixture of (6) (5.96×10^7 d.p.m. of ^3H, spec. act. 2.64×10^7 d.p.m./mg) and [4-^{14}C]sitosterol (7.86×10^6 d.p.m. of ^{14}C, spec. act. 2.85×10^8 d.p.m./mg, the Radiochemical Centre, Amersham) was deposited onto finely grounded oatmeal (800 mg) and fed to 500 young *Tenebrio molitor* larvae (8.0 g) which had been starved for 2 days. After 4 days the larvae were sacrificed, macerated in ethanol, and refluxed for 3 h with 5% ethanolic KOH (100 ml). Ethanol was evaporated under reduced pressure and work-up afforded the unsaponifiable fraction which was benzoylated overnight. Sequential 20% $AgNO_3$–silica gel t.l.c. (hexane–benzene 70 : 30, two elutions) and h.p.l.c. (Varian 5020 L/C instrument, Waters μBondapak-C_{18} column, 3.9 mm × 30 cm, flow rate 2.5 ml/min, solvent methanol–water 97 : 3) yielded pure cholesteryl benzoate which was refluxed for 1 h with 5% ethanolic KOH to yield, after work-up, 3 mg of [23,23,24-$^3H_3$,4-^{14}C]cholesterol (7) (3.95×10^5 d.p.m. of ^{14}C) which was diluted to 1.20 g with cold material.

Methyl 3-*Oxo*[23,23-$^3H_2$, 4-^{14}C]*chol-4-en-24-oate* (15b).—[23,23,24-$^3H_3$, 4-^{14}C]Cholesterol (7) (1.20 g) was submitted to Oppenauer oxidation to give (11b) (900 mg) on which the degradation procedure described above for (11a) was effected. The intermediates of the sequence were crystallized to constant specific activities and $^3H/^{14}C$ ratios (Table 2).

MeOH/MeO^- *Exchange on* (15b).—Compound (15b) (80 mg) was twice exchanged as described above for (15a) to give (16b) (60 mg) purified by preparative t.l.c. (hexane–ethyl acetate, 80 : 20) and crystallized to constant specific activity (Table 2).

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References

1 J. A. Svoboda, M. J. Thompson, W. E. Robbins, and J. N. Kaplanis, *Lipids*, 1978, **13**, 742.
2 F. Nicotra, F. Ronchetti, and G. Russo, *Experientia*, 1978, **34**, 699; F. Nicotra, F. Ronchetti, G. Russo, and L. Toma, *J. Chem. Soc., Chem. Commun.*, 1980, 479; F. Nicotra, P. Pizzi, F. Ronchetti, G. Russo, and L. Toma, *J. Chem. Soc., Perkin Trans. 1*, 1981, 480.
3 P. J. Pettler, W. J. S. Lockley, H. H. Rees, and T. W. Goodwin, *Biochem. J*, 1978, **74**, 397; Y. Fujimoto, N. Awata, M. Morisaki, and N. Ikekawa, *Tetrahedron Lett.*, 1974, 4335.

4 H. L. Holland, *Chem. Soc. Rev.*, 1981, **10**, 435.
5 F. Nicotra, F. Ronchetti, and G. Russo, *Lipids*, 1982, **17**, 184.
6 H. H. Rees, T. G. Davies, L. N. Dinan, W. J. S. Lockley, and T. W. Goodwin, in ' Progress in Ecdysone Research,' ed. J. A. Hoffman, Elsevier/North Holland Biomedical Press, 1980, p. 125.
7 S. Maruyama, Y. Fujimoto, M. Morisaki, and N. Ikekawa, *Tetrahedron Lett.*, 1982, 1701.
8 F. Nicotra, F. Ronchetti, G. Russo, and L. Toma, *Biochem. J.*, 1979, **183**, 495.
9 R. P. Manley, K. W. Curry, N. C. Deno, and M. D. Meyer, *J. Org. Chem.*, 1980, **45**, 4385.
10 E. J. Corey and G. Schmidt, *Tetrahedron Lett.*, 1979, 399.
11 W. C. Still, M. Kahn, and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.
12 R. N. Iacona, A. T. Rowland, and H. R. Nace, *J. Org. Chem.*, 1964, **29**, 3495.

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