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The Effect of Carbon Monoxide on the Nature of the Accumulated 4,4-Dimethyl Sterol Precursors of Cholesterol during its Biosynthesis from [2-^{14}C]Mevalonic Acid *in vitro*

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Cholesterol biosynthesis was studied in rat liver subcellular fractions incubated with DL-[2-^{14}C]mevalonic acid under gas phases consisting of either N_2+O_2 (90:10) or $CO+O_2$ (90:10). CO inhibits cholesterol biosynthesis from [2-^{14}C]mevalonic acid and results in a large accumulation of radioactive 4,4-dimethyl sterols. Separation of the components of the 4,4-dimethyl sterol fraction showed that lanosterol and dihydrolanosterol are the major components that accumulate during cholesterol biosynthesis in an atmosphere containing CO, whereas 14-demethyl-lanosterol and 14-demethyldihydrolanosterol are the major components of the much less intensely radioactive 4,4-dimethyl sterol fraction isolated from incubations with N_2+O_2 as the gas phase. The identities of lanosterol, dihydrolanosterol and 14-demethyldihydrolanosterol were confirmed by both radiochemical and physicochemical methods, including g.l.c. and combined g.l.c.–mass spectrometry. CO therefore results in a qualitative as well as a quantitative difference in the 4,4-dimethyl sterol fraction which arises during cholesterol biosynthesis from mevalonic acid. The specific radioactivity of the [^{14}C]lanosterol biosynthesized in the presence of CO was lower than that of its companion, [^{14}C]dihydrolanosterol. The relative amounts of 4,4-dimethyl-Δ^{24}-sterols and 4,4-dimethyl-24,25-dihydrosterols present in each type of incubation suggest that enzymic reduction of the sterol side chain occurs predominantly at a stage after that of lanosterol.

Olson *et al.* (1957) suggested that during cholesterol biosynthesis, the 14α-methyl group of lanosterol (5α-lanosta-8,24-dien-3β-ol) is lost as CO_2. However, more recently Alexander *et al.* (1972) suggested that this C atom is lost directly from a 14α-aldehyde, as formic acid. Regardless of the oxidation state at which this C atom is removed, the process presumably involves an initial oxidation of the methyl group to a primary alcohol. Gautschi & Bloch (1958) have demonstrated, by elegant radiochemical techniques, that 14-demethyl-lanosterol (4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol; compound 2a, Fig. 1), isolated as 14-demethyldihydrolanosterol (4,4-dimethyl-5α-lanost-8-en-3β-ol; compound 2b, Fig. 1), is a highly radioactive companion of lanosterol during cholesterol biosynthesis from [2-^{14}C]acetate *in vivo*. However, the extremely small amounts of this material precluded any attempt at a physicochemical examination. This and other work (Gautschi & Bloch, 1957, 1958) has established that this compound is most probably a cholesterol biosynthetic intermediate resulting ultimately from the removal of the 14α-methyl group of lanosterol. This process, however, has proved to be of greater complexity than was originally thought, and more recent work has provided strong evidence for the participation of a 4,4-dimethyl-$\Delta^{8,14}$-dienol (4b, Fig. 1) in the overall process by which the 14α-methyl group is lost (Schroepfer *et al.*, 1972; Fiecchi *et al.*, 1972; Akhtar *et al.*, 1972). There is also evidence for the involvement of a 4,4-dimethyl-$\Delta^{8(14)}$-enol (compound 3b, Fig. 1) in this process (Schroepfer *et al.*, 1972). At present, it is difficult to propose a comprehensive sequence leading to the elimination of the 14α-methyl group of lanosterol and involving the participation of all the suggested types of intermediates. In view of the recently acknowledged complexity of this problem, one of the purposes of the present work has been to determine unambiguously the nature of the 4,4-dimethyl sterols and the extent to which they occur during cholesterol biosynthesis from [2-^{14}C]-mevalonic acid.

We have demonstrated (Gibbons & Mitropoulos, 1972) that CO inhibits cholesterol biosynthesis from [2-^{14}C]mevalonic acid *in vitro* and have presented radiochemical evidence for the accumulation of lanosterol and dihydrolanosterol during this process. In the present work we describe the separation of the components of the highly radioactive 4,4-dimethyl sterol fraction biosynthesized under these conditions

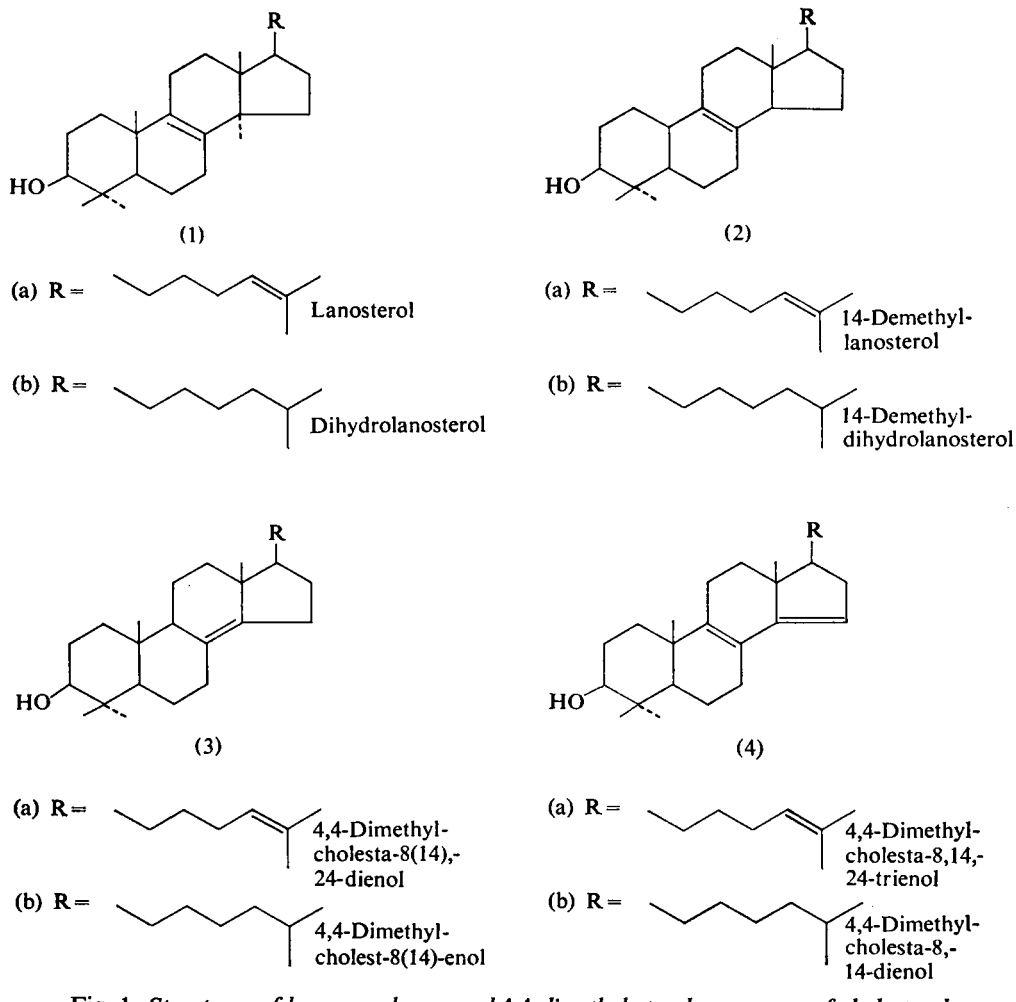


Fig. 1. *Structures of known and proposed* 4,4-*dimethyl sterol precursors of cholesterol*

and provide unequivocal physicochemical and radiochemical evidence for their identity with lanosterol and dihydrolanosterol.

The results are discussed in terms of recent developments concerning the sequence of events leading to the loss of the 14α-methyl group of lanosterol and formation of 14-demethyl-lanosterol during cholesterol biosynthesis.

Experimental

Methods

Preparation of liver cell fractions and incubations. Male rats of the Wistar strain, weighing 125–150g and fed on M.R.C. diet no. 18 (Bruce & Parkes, 1946), were used. The rats were killed by breaking their necks at about 08.00h on the day of the experiment. The livers were removed immediately and, after perfusion with ice-cold 0.25M-sucrose followed by passage through a stainless-steel mincer, a 10000$g_{av.}$ supernatant fraction was obtained by the method of Bucher *et al.* (1959). All subsequent operations before incubation were conducted with the flasks immersed in ice. Incubations were conducted in the manner described by Gibbons & Mitropoulos (1972) by using 33ml of the supernatant fraction obtained at 10000$g_{av.}$ for 20min (S_{10} fraction) in a total volume of 41.5ml of Bucher medium. The appropriate gas mixture (600ml) was bubbled through the incubation mixture and the incubation was started by injecting 1ml of a solution of [2-^{14}C]mevalonic acid (25μCi; 3.62Ci/mol) in 0.1M-potassium phosphate buffer (pH7.4).

Isolation of the 4,4-dimethyl sterol fractions and separation of their components. Each incubation was terminated by the addition of 20vol. of chloroform–methanol (2:1, v/v). The precipitated protein was removed by filtration and the filtrate was washed with 0.2vol. of water. The lower organic phase was evaporated to dryness under reduced pressure and portions were taken both for counting of radioactivity and for analytical t.l.c. followed by radioautography. A portion of the aqueous methanol phase was also removed for counting of radioactivity. The organic extract was chromatographed on ether-washed plates of silica gel H containing Rhodamine 6G (0.5%, w/w) by using chloroform as the developing solvent. The 4,4-dimethyl sterol-containing fraction was eluted from the silica gel with diethyl ether (100ml). A portion of each fraction was taken for counting of radioactivity. To ensure complete removal of contaminating 4α-methyl sterols, the 4,4-dimethyl sterol fraction obtained from the first t.l.c. was rechromatographed in the same system except that Rhodamine 6G was not incorporated into the silica gel. The radioactive band due to the 4,4-dimethyl sterols was located by radioautography (1h exposure) in each case, and the radioactive material was eluted from the plate as described above. A portion was taken for counting of radioactivity. The rest of the fraction was acetylated and chromatographed on a silver nitrate-impregnated plate of silica gel H (10%, w/w; 0.3mm thick) developed with benzene–hexane (1:1, v/v). The radioactive zones of the plate were located by radioautography (1h exposure) and the areas corresponding to the bands of blackening were scraped from the plate. The radioactive materials were eluted with diethyl ether. The radioactive fractions derived from this chromatography and which corresponded to the separated acetates of the 4,4-dimethyl-Δ^{24}- and 4,4-dimethyl-24,25-dihydro sterols from each incubation (four fractions in all) were each rechromatographed on thin-layer plates of alumina G impregnated with silver nitrate (28%, w/w). Plates containing 4,4-dimethyl-Δ^{24}-steryl acetates were developed with benzene–hexane (3:7, v/v) at 5°C; those containing 4,4-dimethyl-24,25-dihydro steryl acetates were developed with benzene–hexane (1:4, v/v) at 5°C. The radioactive bands on each plate were located and eluted as before. Portions of each fraction were removed for counting of radioactivity and for further investigation of their properties by radiochemical and physical techniques.

Gas–liquid chromatography and combined gas–liquid chromatography–mass spectrometry. Analytical g.l.c. was carried out on a Varian Associates dual-channel gas chromatograph series 2700 fitted with flame ionization detectors. Glass columns (6ft × $\frac{1}{8}$in internal diam.) were packed with stationary phases of either 1.5% SE-30 or 1.5% QF-1 coated on Varaport-30 (80–100 mesh). Preparative g.l.c. was carried out on stainless-steel columns (6ft × $\frac{1}{8}$in internal diam.) packed with the same stationary phases as described above. A variable effluent-stream-splitter ensured that 10% of the column effluent passed through the detector whilst the rest was collected and counted for radioactivity. G.l.c.–mass spectrometry was carried out either on a Pye 104 gas chromatograph linked to an A.E.I. MS-902 mass spectrometer or a Varian Associates gas chromatograph series 2400 linked with an A.E.I. MS-12 mass spectrometer.

Assay of radioactivity. Radioactivity was assayed in a Beckman liquid-scintillation spectrometer series LS-250. Counting rates were corrected for quenching by the external-standard ratio method.

Chemical synthesis. 4,4-Dimethylcholesta-5,7-dien-3-one was used as the starting material for the synthesis of those 4,4-dimethyl sterols described below. The dienone was synthesized by Oppenauer oxidation of cholesta-5,7-dienol (Shepherd *et al.*, 1955) followed by methylation (Woodward *et al.*, 1957) of the derived cholesta-4,7-dien-3-one. The physical constants of the 4,4-dimethyldienone were similar to those reported by Gautschi & Bloch (1958).

Preparation of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, 4,4-dimethyl-5α-cholest-8-en-3β-ol and 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol. 4,4-Dimethylcholesta-5,7-dien-3-one (400mg) was reduced with lithium aluminium hydride (100mg) in dry diethyl ether (25ml) to give, on recrystallization, 4,4-dimethylcholesta-5,7-dien-3β-ol (300mg), m.p. 140–141°C [reported, 139–141°C (Gautschi & Bloch, 1958)]. HCl isomerization of the dienol gave, after recrystallization, 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (225mg), $\lambda_{max.}$, 250nm; ϵ_{250}, 17900 litre·mol^{-1}·cm^{-1}; m.p., 142–143°C. The m.p. of the 3-acetate was 154–156°C. The reported physical constants for this material are: m.p., 144–145°C; $\lambda_{max.}$, 248nm; ϵ_{248}, 18600 litre·mol^{-1}·cm^{-1}; m.p. 3-acetate, 151–153°C (Gautschi & Bloch, 1958).

Reduction of the $\Delta^{8,14}$ dienol (300mg) with H_2 and a suspension of Raney nickel in ethanol (Watkinson *et al.*, 1971) gave a mixture of the $\Delta^{8(9)}$-enol and the $\Delta^{8(14)}$-enol in the ratio 7:3 as measured by g.l.c. The mixture was acetylated and recrystallized four times from chloroform–methanol to give plate-like crystals of m.p. 120–122°C [reported, 122–124°C (Gautschi & Bloch, 1958)]. The $\Delta^{8(9)}$ position of the double bond was confirmed by the fact that the ϵ_{215} was 5150 litre·mol^{-1}·cm^{-1} as opposed to ϵ_{215} of 7380 litre·mol^{-1}·cm^{-1} for 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol (Bladon *et al.*, 1952). There was no absorption at 250nm. Reduction of 4,4-dimethylcholesta-5,7-dien-3-one (350mg) with H_2 and Adams catalyst (PtO_2) by a modification of the method of Gautschi & Bloch (1958) gave 4,4-dimethyl-5α-cholest-8(14)-enol. After crystallization, the material (230mg) exhibited no dienic absorption at 283nm but showed a greater than usual intensity

of absorption in the 215nm region (ϵ_{215}, 7380 litre·mol^{-1}·cm^{-1}). After two further recrystallizations from methanol, the melting point was 143–144°C and that of the acetate was 115–116°C [reported 142–143°C and 115–116°C respectively (Gautschi & Bloch, 1958)]. The 3-one had m.p. 94–96°C [reported 98–99°C (Fried & Brown, 1967)]. All melting points are uncorrected. Extinction coefficients were determined in ethanol.

Materials

NAD^+ and fructose 1,6-diphosphate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Silica gel H was obtained from E. Merck A.G., Darmstadt, Germany, and was washed twice with ethanol and once with ether before use. Alumina type H was obtained from Laporte Industries Ltd., London W.1, U.K. Alumina G for t.l.c. was obtained from M. Woelm, Eschwage, Germany. Cholesta-5,7-dienyl benzoate was obtained from Ralph Emanuel Ltd., Wembley, Middlesex, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. DL-[2-^{14}C]Mevalonic acid lactone (7.1 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was diluted with non-radioactive DL-mevalonic acid lactone (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.). Benzene, hexane, ethanol, methanol and chloroform were all commercial AnalaR-grade preparations and were distilled immediately before use. Diethyl ether (ether) was purified on a column of alumina type H before use.

Lanosterol (m.p. 136–138°C) and cholesterol (m.p. 148–148.5°C) were obtained from commercial preparations as described by Gibbons & Mitropoulos (1972). 24,25-Dihydrolanosterol (m.p. 144–145.5°C) was prepared by catalytic hydrogenation of the acetylated commercial lanosterol (Barton, 1951) followed by crystallization from chloroform–methanol and subsequent alkaline hydrolysis.

Results

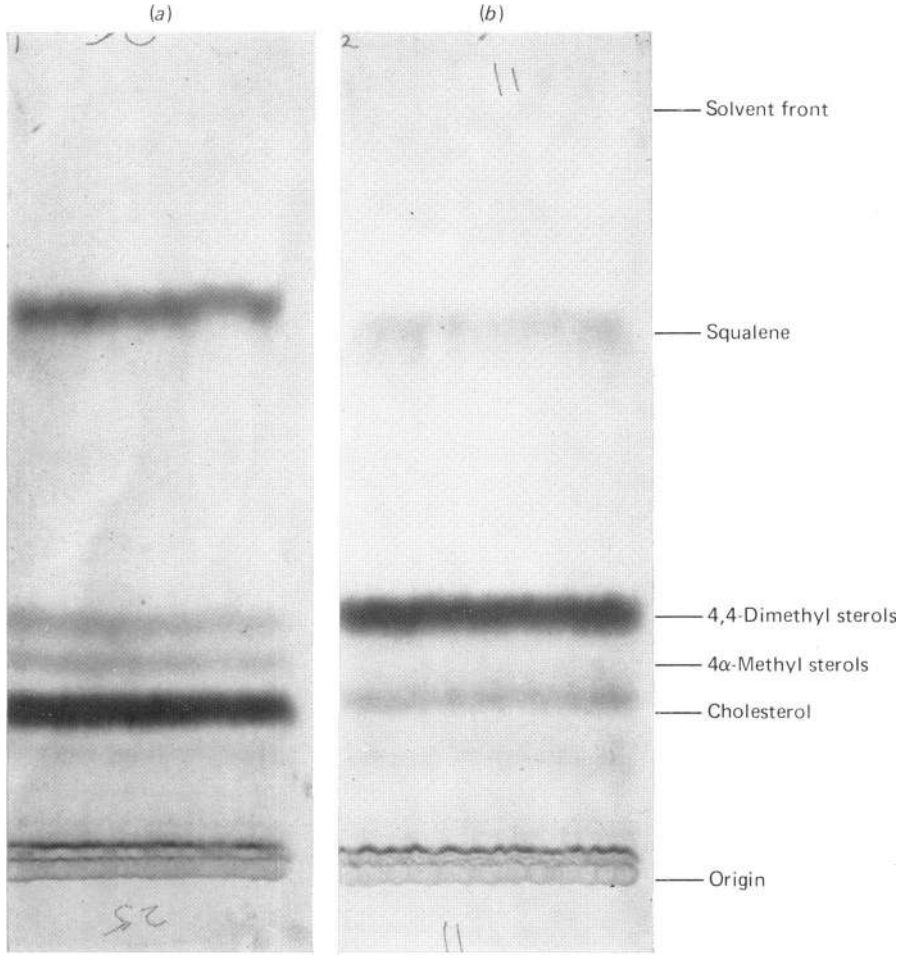
A 10000$g_{av.}$ supernatant fraction of rat liver homogenate was incubated with [2-^{14}C]mevalonic acid under an atmosphere of N_2+O_2 (incubation 1) for 1h. A similar incubation was conducted under $CO+O_2$ (incubation 2). The amounts of radioactivity incorporated into the total lipid fractions were almost identical [20.736×10^6 d.p.m. (incubation 1) and 19.360×10^6 d.p.m. (incubation 2)]. A radioautograph of portions of the radioactive lipid fractions after chromatography on silica gel H is shown in Plate 1 and shows a typical effect of CO on the distribution of radioactivity from [2-^{14}C]mevalonic acid in cholesterol and its triterpenoid precursors. There was a large decrease in the amount of radioactivity that appeared in the cholesterol and 4α-methyl sterol fractions, concomitant with a large accumulation of radioactive 4,4-dimethyl sterols. After preparative chromatography of the rest of the lipid fractions, the 4,4-dimethyl sterol fraction from incubation 1 contained 5.899×10^6 d.p.m. whereas that from incubation 2 contained 11.928×10^6 d.p.m. Subsequent chromatography of these fractions in the same system completely eliminated 4α-methyl sterols, as judged by radioautography. The purified 4,4-dimethyl sterols so obtained (R_F 0.38) contained 2.263×10^6 d.p.m. (incubation 1) and 9.411×10^6 d.p.m. (incubation 2).

After acetylation of the 4,4-dimethyl sterol fraction and chromatography on silica gel–silver nitrate t.l.c. plates followed by radioautography, the radioautographs appeared as shown in Plate 2. Bands A and B correspond to the acetates of 4,4-dimethyl-24,25-dihydro sterols and 4,4-dimethyl-Δ^{24}-sterols respectively. Bands 1C and 2C are in the region where the acetates of conjugated dienic sterols would occur. The double bands represented by 1D and 2D were later shown to be due to unacetylated Δ^{24} and 24,25-dihydro sterols. The radioactivities of each of these bands are given in Table 1 and Plate 2.

Each band A (1A and 2A, containing the 4,4-dimethyl-24,25-dihydro steryl acetates isolated from incubations under gas phases of N_2+O_2 and $CO+O_2$ respectively) was finally chromatographed on a silver nitrate-impregnated plate of alumina. Bands 1B (containing the 4,4-dimethyl-Δ^{24}-steryl acetates isolated from the N_2+O_2 incubation) and 2B (containing those derived from the incubation under $CO+O_2$) were chromatographed in a similar way. Table 1 shows the distribution of radioactivity (located by radioautography) in the various zones of each plate, and the probable chromatographic identity of each fraction. In the incubation under $CO+O_2$, 87.7% of the total radioactivity was in material with the chromatographic properties of 14α-methyl sterols of the lanosterol type. Only 5.2% had the properties of 14-demethyl sterols. The constituents of the much less radioactive fraction biosynthesized in the presence of N_2+O_2 were predominantly materials with the chromatographic properties of 14-demethyl sterols (76.2% of the total radioactivity) such as 4,4-dimethylcholesta-8,24-dienol and its 24,25-dihydro derivative. Only 5.8% of the total radioactivity was due to 14α-methyl sterols. There was no indication of the presence of $\Delta^{8(14)}$ or Δ^7 sterols in either case.

Further analysis of the separated 4,4-dimethyl steryl acetates

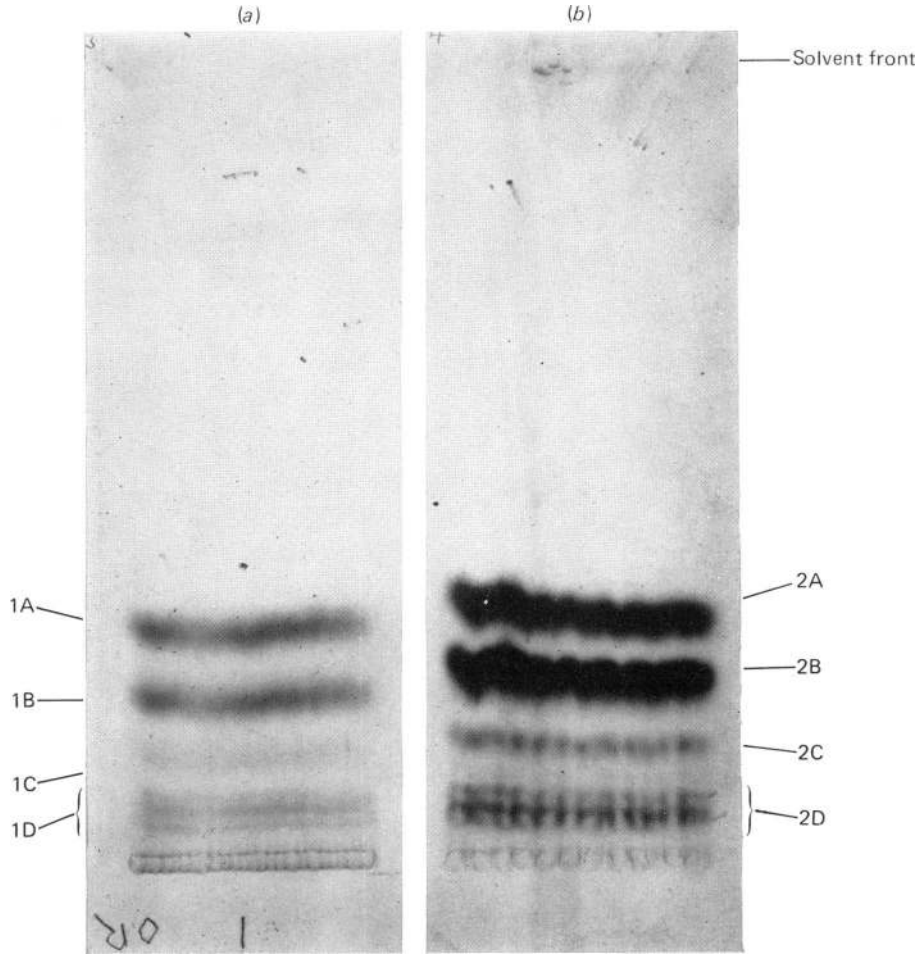
Analytical g.l.c. of fractions from the silica gel–silver nitrate chromatograms. G.l.c. of the radioactive bands with the chromatographic mobilities of



EXPLANATION OF PLATE 1

Radioautograph showing the distribution of radioactivity in cholesterol and its triterpenoid precursors after incubation of a rat liver fraction (10000$g_{av.}$ *for* 20 *min*) *with* [2-^{14}C]*mevalonic acid in the presence of* N_2+O_2 (*incubation* 1) *or* $CO+O_2$ (*incubation* 2)

Portions of the radioactive lipid fractions (172800 d.p.m. from incubation 1; 161300 d.p.m. from incubation 2) were chromatographed on plates of silica gel H containing Rhodamine 6G. Before chromatography, marker compounds of cholesterol (200 μg) and lanosterol (400 μg) were added to each portion. The radioactive bands were located by radioautography and two of these corresponded exactly to the marker cholesterol and lanosterol. (*a*) Incubation 1; (*b*) incubation 2.



EXPLANATION OF PLATE 2

Radioautographs showing the distribution of radioactivity in the 4,4-*dimethyl steryl acetate fractions derived from incubation of rat liver fractions* ($10000g_{av.}$ *for* 20 *min*) *with* [2-^{14}C]*mevalonic acid under* N_2+O_2 (*incubation* 1) *or* $CO+O_2$ (*incubation* 2)

The 4,4-dimethyl steryl acetate fractions were obtained after acetylation of the 4,4-dimethyl sterol fractions from t.l.c. on silica gel H. Each fraction was chromatographed on plates of silver nitrate-impregnated silica gel H (10% $AgNO_3$, w/w) by using benzene–hexane (1:1, v/v) as the developing solvent. Radioactive zones were located by radioautography and the areas of the plate corresponding to the zones of blackening on the radioautograph were eluted with diethyl ether. Portions were taken for radioassay, and the radioactivities of the fractions were as follows ($10^{-6}\times$d.p.m.): 1A, 0.6355; 1B, 0.6461; 1C, 0.1519; 1D, 0.4884; 2A, 2.832; 2B, 4.319; 2C, 0.6080; 2D, 1.369. (*a*) Incubation 1; (*b*) incubation 2.

Table 1. *Radioactivities and chromatographic identities of the acetates of* 4,4-*dimethyl sterols biosynthesized from* [2-^{14}*C*]*mevalonic acid in the presence of* N_2+O_2 (*incubation* 1) *and* $CO+O_2$ (*incubation* 2)

Fractions 1A–2B were obtained after chromatography of the acetylated 4,4-dimethyl sterol fractions on silica gel impregnated with $AgNO_3$ as described in the Experimental section. Fractions designated 'A' contain one nuclear double bond and a saturated side chain. Those designated 'B' contain one nuclear double bond plus a Δ^{24} bond. Each of these fractions was re-chromatographed on alumina impregnated with $AgNO_3$. The fractions derived from this chromatography are listed in order of increasing polarity; thus fraction $1B_I$ is less polar than $1B_{III}$.

Incubation	Fraction from silica gel–$AgNO_3$ chromatography	Sterol type	Radioactivity (d.p.m.)	Fraction from alumina–$AgNO_3$ chromatography	Radioactivity (d.p.m.)	Probable identity
N_2+O_2	1A	24,25-Dihydro	635500	$1A_I$	519000	4,4-Dimethylcholest-8-enyl acetate
				Rest of plate	91300	—
	1B	Δ^{24}	646100	$1B_I$	26300	?
				$1B_{II}$	56300	Lanosteryl acetate
				$1B_{III}$	277800	4,4-Dimethylcholesta-8,24-dienyl acetate
$CO+O_2$	2A	24,25-Dihydro	2832000	$2A_I$	2167000	Dihydrolanosteryl acetate
				$2A_{II}$	329000	4,4-Dimethylcholest-8-enyl acetate
	2B	Δ^{24}	4319000	$2B_I$	270800*	Dihydrolanosteryl acetate
				$2B_{II}$	3431000	Lanosteryl acetate
				$2B_{III}$	63100	4,4-Dimethylcholesta-8,24-dienyl acetate
				$2B_{IV}$	125000	?

* This band most probably represents contamination of the 4,4-dimethyl-Δ^{24}-steryl acetate fraction (2B) by 24,25-dihydro steryl acetates during chromatography on silver nitrate–silica gel.

Table 2. *Gas–liquid chromatography of the acetates of the* 4,4-*dimethyl sterols biosynthesized by fractions of rat liver homogenates* ($10000g_{av.}$ *for* 20 *min*) *in the presence of* [2-^{14}*C*]*mevalonic acid*

Each fraction was obtained by t.l.c. on silica gel H and silica gel–silver nitrate only (fractions C and D) or silica gel H and silica gel–silver nitrate followed by alumina–silver nitrate (fractions A and B). The retention times relative to cholesterol of those compounds marked † are calculated, as authentic reference materials were unavailable. All authentic materials, and those biosynthetic fractions marked ‡, were chromatographed in duplicate, the reported relative retention times being an average of the two values. The g.l.c. conditions were: column temperature, 206°C; detector, 273°C; injector, 263°C; N_2 flow rate, 30ml/min; stationary phase, 1.5% QF-1. Values in parentheses after the compounds present in fractions D refer to the proportion of that component as a percentage of the total peak area. The most important 4,4-dimethyl steryl acetates obtained from each incubation are underlined.

Fractions isolated from incubations under N_2+O_2

T.l.c. fraction*	Relative retention time of peaks	Probable identity	% difference from relative retention time of authentic compound
$1A_I$‡	2.531	4,4-Dimethylcholest-8-enyl acetate	+0.5
$1B_{II}$	2.884	Lanosteryl acetate	−0.2
$1B_{III}$	1.622, 2.766	?+4,4-Dimethylcholesta-8,24-dienyl acetate	+1.6
1D‡	1.420, 1.506	4,4-Dimethylcholest-8-enol (54.8%)	+0.3
		4,4-Dimethylcholesta-8,24-dienol (45.2%)	−1.4

Fractions isolated from incubations under $CO+O_2$

$2A_I$‡	2.690	24,25-Dihydrolanosteryl acetate	+0.6
$2A_{II}$	2.465	4,4-Dimethylcholest-8-enyl acetate	−2.1
$2B_I$	2.601, 2.669	?+24,25-Dihydrolanosteryl acetate	−0.2
$2B_{II}$‡	2.875	Lanosteryl acetate	−0.6
2C	1.445, 1.530	?+?	
2D‡	1.446, 1.545	24,25-Dihydrolanosterol (35.8%)	+0.3
		Lanosterol (64.2%)	−0.7

Authentic material	Relative retention time
Lanosterol	1.556
24,25-Dihydrolanosterol	1.441
4,4-Dimethylcholest-8-enol	1.415
4,4-Dimethylcholesta-8,24-dienol†	1.527
Lanosteryl acetate	2.891
24,25-Dihydrolanosteryl acetate	2.675
4,4-Dimethylcholest-8-enyl acetate	2.519
4,4-Dimethylcholest-8(14)-enyl acetate	2.350
4,4-Dimethylcholesta-8,14-dienyl acetate	2.381
4,4-Dimethylcholesta-8,24-dienyl acetate†	2.722
4,4-Dimethylcholesta-8(14),24-dienyl acetate†	2.539
4,4-Dimethylcholesta-8,14,24-trienyl acetate†	2.573

* Fractions refer to Plate 2 and Table 1.

unacetylated sterols (bands 1D and 2D, Plate 2) each gave two peaks with retention times very close to those of the authentic unacetylated sterols (Table 2). G.l.c. of the material with the chromatographic mobility of a 4,4-dimethyl conjugated diene acetate derived from the incubation under $CO+O_2$ (fraction 2C, Plate 2) showed one peak and a shoulder of shorter retention time. However, these retention times were more typical of 3β-alcohols than of acetates.

Analytical g.l.c. and g.l.c.–mass spectrometry of the purified 4,4-*dimethyl steryl acetate fractions obtained after alumina–silver nitrate chromatography.* The most intensely radioactive 4,4-dimethyl steryl acetate fraction from the incubation under N_2+O_2 ($1A_I$) had the chromatographic properties of 4,4-dimethylcholest-8-enyl acetate. G.l.c. of this fraction showed the presence of only one component, with a relative retention time almost identical with that of an

authentic sample. On analysis by g.l.c.–mass spectrometry, the mass spectrum showed a molecular ion at 456. The measured mass was 456.3958 (calculated for $C_{31}H_{52}O_2$, 456.3967). This was also the base peak in the spectrum. Other ions were observed at *m/e* 441 (M–CH_3, 18% of base peak); 396 (M–acetate, 16.5%), 381 [M–(CH_3+acetate), 27%], 343 (M–side chain, 11.5%), 301 [M–(side chain+part of ring D), 5%] and 283 [M–(side chain+acetate), 11.5%]. The spectrum was virtually identical with that of a chemically synthesized sample of 4,4-dimethylcholest-8-enyl acetate and was similar to a published spectrum of this compound (Galli & Maroni, 1967). G.l.c. of the other major radioactive component ($1B_{III}$) of the 4,4-dimethyl steryl acetate fraction gave a peak with a retention time almost identical with that calculated for 4,4-dimethylcholesta-8,24-dienyl acetate. However, another peak of shorter retention time (Table 2) was also present and may have been due to breakdown of this compound. G.l.c. of the other, minor component ($1B_{II}$) gave a peak with a relative retention time very close to that of lanosteryl acetate.

G.l.c. of the major components of the 4,4-dimethyl steryl acetate fraction isolated from the incubation under $CO+O_2$ ($2A_I$ and $2B_{II}$) showed only one peak in each case with retention times almost identical with those observed for dihydrolanosteryl acetate and lanosteryl acetate respectively. In addition, analysis by g.l.c.–mass spectrometry of the former compound gave ions at *m/e* 455 (M–CH_3, 23.5% of base peak), 410 (M–acetate, 6.3%), 395 [M–(CH_3+acetate), 100%, base peak], 355 [M–(side chain+2H), 3.4%], 297 [M–(side chain+acetate), 6.8%] and 255 [M–(side chain+acetate+part of ring D), 8.1%]. No molecular ion was produced. The spectrum was virtually superimposable on the spectrum of authentic dihydrolanosteryl acetate. G.l.c.–mass spectrum analysis of the compound with the chromatographic properties of lanosteryl acetate gave a mass spectrum with a molecular ion at *m/e* 468, 6.7%. Other ions appeared at *m/e* 453 (M–CH_3, 23.8%), 408 (M–acetate, 6.5%), 393 [M–(CH_3+acetate), 62.7%], 355 [M–(side chain+2H), 1.0%], 339 [M–(acetate+part of side chain), 3.9%], 297 [M–(acetate+side chain), 2.6%] and 255 [M–(acetate+side chain+part of ring D), 8.2%]. The base peak in the spectrum occurred at *m/e* 69 and was presumably due to the loss of a C_5H_9 fragment that arose during allylic cleavage of the Δ^{24}-bond-containing side chain. Again, the mass spectrum was virtually identical with that of authentic lanosteryl acetate. G.l.c. of the other minor components of this 4,4-dimethyl steryl acetate fraction gave the expected retention times (Table 2).

Preparative g.l.c. Portions of the fractions possessing the chromatographic and physical properties of 4,4-dimethylcholest-8-enyl acetate ($1A_I$) from the incubation under N_2+O_2 and lanosteryl acetate ($2B_{II}$) and dihydrolanosteryl acetate ($2A_I$) from the incubation under $CO+O_2$ were analysed further by preparative g.l.c. To a portion of fraction $1A_I$ (5200d.p.m.) was added non-radioactive carrier material (10μg) and cholesterol (3μg) as internal standard. Fractions were collected at timed intervals as shown in Fig. 2(*a*). The peak for mass was very close to the peak for radioactivity, the latter having a retention time 10–40s shorter than that of the mass peak. This small discrepancy was probably due to isotopic fractionation, a phenomenon that has been observed, not only on g.l.c. (Gunter & Gleason, 1971), but also during adsorption chromatography (Paliokas & Schroepfer, 1968).

To portions of fractions $2A_I$ (21000d.p.m.) and $2B_{II}$ (68000d.p.m.) was added 16μg of a mixture of non-radioactive lanosteryl acetate and dihydrolanosteryl acetate. Cholesterol (3μg) was also added to each sample as internal standard. After chromatography, the profiles for radioactivity were almost, but not quite, superimposable on the respective mass profiles (Figs. 2*b* and 2*c* respectively). The slight deviation may be ascribed to the phenomenon of isotopic fractionation mentioned above.

Isotope dilution analysis. Portions of radioactive fraction $1A_I$ were added independently to larger weights of non-radioactive dihydrolanosteryl acetate, 4,4-dimethylcholest-8(14)-enyl acetate and 4,4-dimethylcholest-8-enyl acetate. Each mixture was recrystallized several times from chloroform–methanol. Only in the case of the latter compounds did the specific radioactivity remain constant during recrystallization (initially 705.0, then 641.7, 624.6, 618.9 and 641.7d.p.m./mg). Recrystallization of the other samples resulted in a large decrease in the specific radioactivity of each successive crop of crystals. To fraction $1B_{II}$ (one of the minor constituents of the 4,4-dimethyl steryl acetate mixture that arose from the incubation under N_2+O_2) was added non-radioactive lanosteryl acetate to give an initial specific radioactivity of 1692d.p.m./mg and the sample was recrystallized. After an initial small decrease, there was no significant decline in specific radioactivity after further recrystallizations (1255, 1108, 1107 and 1132d.p.m./mg). Fractions $2A_I$ and $2B_{II}$ showed little or no decrease in specific radioactivity after addition of non-radioactive dihydrolanosteryl acetate (initially 1516d.p.m./mg) and lanosteryl acetate (initially 2164d.p.m./mg) respectively, followed by four recrystallizations in each case (1403, 1322, 1368 and 1353d.p.m./mg, and 2196, 2105, 2132 and 2035d.p.m./mg respectively).

Quantitative aspects of 4,4-*dimethyl sterol metabolism*

The chemical and radiochemical purity of the various isolated 4,4-dimethyl steryl acetate fractions made possible a reasonably accurate measurement of

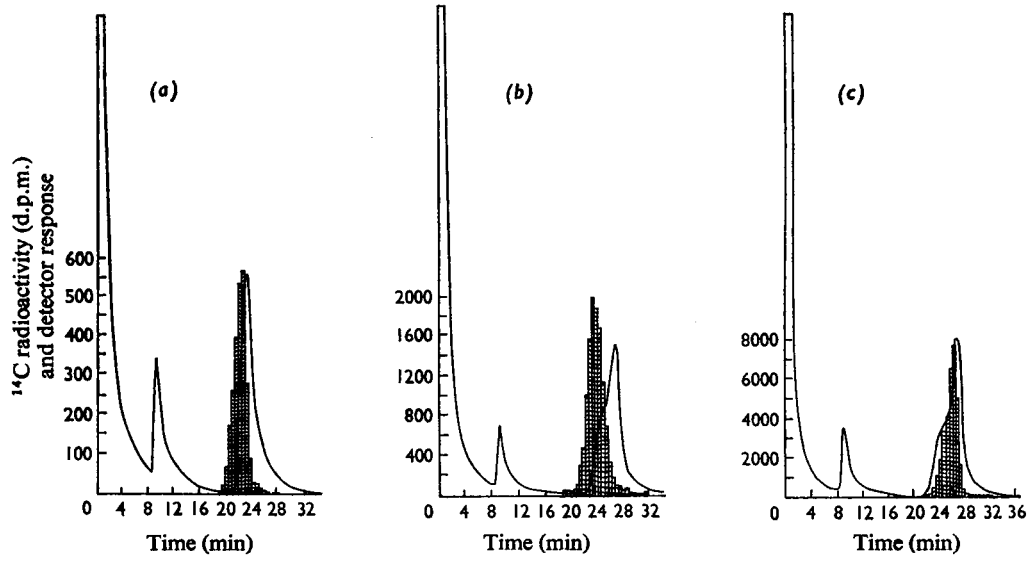


Fig. 2. *Preparative g.l.c. of radioactive* 4,4-*dimethyl steryl acetates isolated from incubations of rat liver homogenate fractions* (10000$g_{av.}$ *for* 20 *min) with* [2-^{14}C]*mevalonic acid under* N_2+O_2 (2*a*) *or* $CO+O_2$ (2*b and* 2*c*)

During chromatography of 2(*a*), 2(*b*) and 2(*c*), fractions were taken every 4 min for the first 16 min. The next three fractions were taken at 1 min intervals and subsequent fractions were taken at intervals of 30 s. Each fraction was collected in a glass capillary tube at ambient temperature and the condensed radioactive materials were washed into scintillation vials with diethyl ether (1 ml). Each fraction was assayed for radioactivity. G.l.c. operating conditions were: column temperature, 204°C; injector, 255°C; detector, 270°C; N_2 flow rate, 30 ml/min. QF-1 (1.5%) was used as the stationary phase. Radioactivity is indicated by the hatched regions.

the absolute amounts present after incubation and of their specific radioactivities by quantitative g.l.c. The instrument was calibrated, in terms of detector response per unit weight of steryl acetate chromatographed, for lanosteryl acetate, dihydrolanosteryl acetate and 4,4-dimethylcholest-8-enyl acetate.

Known portions of the ^{14}C-labelled 4,4-dimethylcholest-8-enyl acetate fraction (isolated from the incubation under N_2+O_2) and of the [^{14}C]lanosteryl acetate and [^{14}C]dihydrolanosteryl acetate fractions (isolated from the incubation under $CO+O_2$) were chromatographed and the peak areas compared with those obtained above. The absolute weight of ^{14}C-labelled 4,4-dimethylcholest-8-enyl acetate isolated was 6.86 μg (0.015 μmol) of specific radioactivity 75600 d.p.m./μg (3.447×10^7 d.p.m./μmol). Adjusting for losses during the chromatographic procedures, the total weight of ^{14}C-labelled 4,4-dimethylcholest-8-enol present at the end of the incubation was 0.030 μmol. Similarly, the amount of [^{14}C]lanosteryl acetate isolated from the incubation under $CO+O_2$ was 50.5 μg (0.108 μmol) of specific radioactivity 68000 d.p.m./μg (3.182×10^7 d.p.m./μmol). After adjustment for losses, this corresponds to a total amount of 0.156 μmol of [^{14}C]lanosterol present at the end of the incubation. The corresponding values for [^{14}C]-dihydrolanosteryl acetate are: 23.2 μg (0.049 μmol), specific radioactivity 93400 d.p.m./μg (4.390×10^7 d.p.m./μmol). The total amount of [^{14}C]dihydrolanosterol present at the end of the incubation was 0.073 μmol. If, during the incubations, each of these compounds had been biosynthesized exclusively from the exogenously added [2-^{14}C]mevalonic acid (specific radioactivity 7.964×10^6 d.p.m./μmol) then the specific radioactivities of these 4,4-dimethyl steryl acetates should have been 4.780×10^7 d.p.m./μmol. The significance of these observed and calculated values are discussed below.

Discussion

Lanosterol and dihydrolanosterol have been identified as the major 4,4-dimethyl sterols which accumulate during cholesterol biosynthesis in an atmosphere containing CO and O_2. 14-Demethyldihydrolanosterol (4,4-dimethylcholest-8-enol) and 14-demethyllanosterol (4,4-dimethylcholesta-8,24-dienol) are the major constituents of the much smaller 4,4-dimethyl sterol fraction formed during cholesterol biosynthesis in the presence of N_2 and O_2. The present results pro-

vide a complete physicochemical and radiochemical identification of the first three compounds. Lack of available authentic material has prevented such a rigorous identification of the material proposed to be 14-demethyl-lanosterol. Nevertheless, the chromatographic properties of the alcohol and its acetate render it very unlikely that this compound differs in structure from that proposed above.

To our knowledge, this is the first combined physicochemical and radiochemical identification of 4,4-dimethylcholest-8-enol in rat liver actively biosynthesizing cholesterol in the absence of inhibitors. However, Scallen *et al.* (1971) have identified this compound, which accumulates during inhibition of cholesterol biosynthesis *in vitro* by cholestan-3β,5α,6β-triol.

4,4,14α-Trimethyl-Δ^8 sterols, of the type that accumulate under $CO+O_2$, constitute a very small amount (less than 6%) of the diminished total radioactivity of the 4,4-dimethyl sterol fraction isolated from the incubation conducted under N_2 and O_2. The total amount of lanosterol and dihydrolanosterol present after incubation under $CO+O_2$ is therefore some 90–100 times greater than that observed after a similar incubation under N_2+O_2. Therefore CO is an extremely potent inhibitor of some reaction, presumably immediately after the formation of lanosterol, the ultimate result of which is removal of the 14α-methyl group. Initially it may appear that CO affects the initial hydroxylation of the 14α-methyl group. However, the possibility that a CO-inhibitable 15-hydroxylation is a prerequisite for initiation of oxidation of the 14α-methyl group cannot be overlooked. A hydroxylation of this type, although utilizing different potential precursors of cholesterol, has been suggested by Schroepfer *et al.* (1972). The presence of a 3-hydroxy group is known to be required for hydroxylation of the 4α-methyl group of 4,4-dimethylcholest-7-enol and 4α-methylcholest-7-enol (Swindell & Gaylor, 1968). The corresponding 3-ketones are not hydroxylated. In the present case retention of a 15-hydroxy group until the 14α-methyl has been fully oxidized to a carboxyl group could lead to the concerted loss of the carboxyl and hydroxyl functions concomitant with the introduction of a Δ^{14} bond. This would result in the formation of a 4,4-dimethyl-$\Delta^{8,14}$-diene, a proposed precursor of cholesterol (Akhtar *et al.*, 1972; Fiecchi *et al.*, 1972).

No radioactive 4,4-dimethyl-$\Delta^{8(14)}$ sterols were detected in either type of incubation. If this type of compound is indeed a precursor of cholesterol, then its rate of turnover must be so rapid as to escape detection by the methods employed in this investigation (the limit of sensitivity is 0.1–0.2μg). A compound that had the properties on t.l.c. of a 4,4-dimethyl-$\Delta^{8,14}$-diene acetate constituted a significant proportion of the 4,4-dimethyl steryl acetate fraction from both the $CO+O_2$ incubation (6.5%) and the N_2+O_2 incubation (8.0%). However, in the former case, the retention time on g.l.c. did not correspond to that of any available authentic material.

During this investigation, some other interesting observations were made concerning the relative amounts and specific radioactivities of the 4,4-dimethyl sterols identified after incubation with [2-^{14}C]mevalonic acid, and may have some bearing on the major pathway of cholesterol biosynthesis from lanosterol. First, the ratio of dihydrolanosterol to lanosterol is smaller than that of 14-demethyldihydrolanosterol to 14-demethyl-lanosterol. Possibly dihydrolanosterol was present in considerable quantity in the inhibited incubation only because the rate of oxidation of lanosterol was decreased, providing a virtually static pool of lanosterol for reduction of the Δ^{24} bond by an enzyme that does not normally utilize lanosterol as a substrate. This may mean that 14-demethyl-lanosterol is a better substrate for the Δ^{24} reductase enzyme than lanosterol itself or that 14-demethyl-lanosterol is metabolized at a faster rate than 14-demethyldihydrolanosterol. In either case, the present results indicate that 14-demethyl-lanosterol is a more important precursor of cholesterol than dihydrolanosterol. Also, during the incubation under N_2+O_2, although a significant amount of [^{14}C]lanosterol was isolated, [^{14}C]dihydrolanosterol could not be detected. This result could be due to a rapid rate of turnover of [^{14}C]dihydrolanosterol but this explanation is inconsistent with the observation that when the rate of further metabolism of the trimethyl sterols is decreased (during CO inhibition of cholesterol biosynthesis), dihydrolanosterol still accumulates to a smaller extent than lanosterol, even though reduction remains the major biochemical outlet for lanosterol. Also, we have shown (G. F. Gibbons & K. A. Mitropolous, unpublished work) that lanosterol is converted into cholesterol at a rate approximately twice that of dihydrolanosterol.

Secondly, the specific radioactivity of lanosterol is lower than that of dihydrolanosterol. This is interpreted as being due to the presence of a non-metabolically active pool of lanosterol consisting of about 17.9μg (0.042μmol) present in the rat liver before incubation. This type of problem was encountered by Popják (1954) in studies on cholesterol biosynthesis, and a relatively large pool of lanosterol has been demonstrated in rat skin (Clayton *et al.*, 1963).

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