(17) C. Kemal, R. F. Williams, T. W. Chan, and T. C. Bruice, *J. Am. Chem. Soc.*, submitted for publication.
(18) S. Ghisla, W. Hartmann, P. Hemmerich, and F. Müller, *Justus Liebigs Ann. Chem.*, 1388 (1973).
(19) G. Blankenhorn, S. Ghisla, and P. Hemmerich, *Z. Naturforsch, Teil B*, **27**, 1038 (1972).
(20) C. Kemal and T. C. Bruice, *J. Am. Chem. Soc.*, **98**, 3955 (1976).
(21) D. Clerin and T. C. Bruice, *J. Am. Chem. Soc.*, **96**, 5571 (1974).
(22) C. Kemal and T. C. Bruice, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 995 (1976).
(23) T. H. Fife and E. Anderson, *J. Org. Chem.*, **36**, 2357 (1971).
(24) J. N. S. Tam, G. N. Vaughan, M. P. Mertes, G. S. Rork, and I. H. Pitman, *J. Am. Chem. Soc.*, in press.
(25) T. C. Bruice, *Prog. Bioorg. Chem.*, **4**, 1 (1976).
(26) E. Hayon, J. Bata, N. N. Lichtin, and M. Simic, *J. Phys. Chem.*, **76**, 2072 (1972).
(27) Due to a misunderstanding on our part in the comparison of the $E^{\circ 1}$ values of Rao and Hayon (ref 5) and Lilie, Beck, and Henglein (ref 29) the sign for the $E^{\circ 1}$ values for $\cdot CH_2O^-$ and $\cdot CH_2OH$ were inverted in ref 10. This resulted in the calculated value of ΔG° for formation of $\cdot CH_2OH$ from CH_2O being ca. 6–8 kcal M^{-1} greater than the correct value (i.e., the radical pairs ~FlH·$_T$·CH_2OH~ and ~FlH·$_T$·CH_2O^-~ are correspondingly lowered).
(28) V. J. Lilie, G. Beck, and A. Henglein, *Ber. Bunsenges. Phys. Chem.*, **75**, 458 (1971).

Carbon-13 NMR Studies on Cholesterol Biosynthesized from [^{13}C]Mevalonates

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Abstract: Two specimens of [^{13}C]cholesterol, one biosynthesized from [5-^{13}C]mevalonate and the other from [3′,4-$^{13}C_2$]mevalonate, were examined by Fourier transform ^{13}C NMR spectroscopy. The observations confirmed the biochemically predictable positions of ^{13}C labels in cholesterol and provided also an unambiguous proof of the assignments of the ^{13}C resonances for 16 of the 27 carbon atoms in the ^{13}C NMR spectrum of cholesterol. New and additional evidence is provided for a 1:2 methyl shift, from position 14 to 13, during the cyclization of squalene oxide to lanosterol. The stereochemistry of the reduction of the 24(25) double bond in the side chain of lanosterol (or of desmosterol), determined by others, and the magnetic nonequivalence of C-26 and C-27 in cholesterol led to the assignment of the resonance at 22.9 ppm downfield from the reference to C-27 (derived from C-3′ of mevalonate), the pro-(*S*)-methyl group attached to C-25. The resonance of C-26, the pro-(*R*)-methyl group attached to C-25, originating from C-2 of mevalonate, is at 22.7 ppm downfield from the reference. One-bond (sp^3) and triple-bond (vicinal) ^{13}C–^{13}C coupling constants are also reported.

During the earliest explorations of cholesterol biosynthesis in the 1950's the time-consuming carbon-by-carbon degradations of cholesterol[2] or squalene biosynthesized from [^{14}C]acetates[3a] or [^{14}C]mevalonate[3b,c] were the only means available to establish overall biosynthetic pathways. The availability of ^{13}C NMR would reduce today similar studies to a few weeks instead of the years of effort needed earlier.

The intermediary steps in the biosynthesis of cholesterol starting from acetyl-CoA are well known and the positions of the two carbon atoms of acetate in the sterol have been completely mapped.[2] When, after the discovery of mevalonic acid as an intermediate in sterol biosynthesis by Tavormina et al.,[4] Cornforth et al.[3b] determined the distribution of mevalonate carbons in squalene, they also predicted the probable distribution of the carbon atoms of this intermediate in lanosterol and cholesterol. Isler et al.[5] have shown that C-2 of mevalonate was found at C-7, C-22, and at either C-26 or C-27 of cholesterol, in accord with the prediction.

Although the distribution of the carbon atoms of mevalonate in cholesterol has been determined for only a few positions, the total pattern cannot be in doubt because (a) of the established pattern of acetate carbons in cholesterol; (b) of the known biosynthesis of mevalonate from acetyl-CoA through 3-hydroxy-3-methylglutaryl-CoA; (c) of the well-documented biosynthesis of squalene from mevalonate; and (d) because the principal postulates of the "biogenetic isoprene rule", pertaining to the cyclization of squalene (2,3-dioxide) to lanosterol, formulated on theoretical grounds by Eschenmoser et al.,[6b] following the proposals of Woodward and Bloch,[6a] have been proved also. The "biogenetic isoprene rule" requires—among other things—two 1:2 methyl migrations during the cyclization of squalene to lanosterol: one from C-14 to C-13 and the other from C-8 to C-14. The first of these was proved by Cornforth et al.[7] with the aid of [3′,4-$^{13}C_2$]mevalonate and the second by Maudgal et al.[8] with the aid of a synthetic all-trans [^{13}C]squalene. The first of these methyl migrations is of particular interest because it occurs within one specific isoprenoid unit of squalene and results in two ^{13}C atoms being bonded together (C-18/C-13) in cholesterol when [3′,4-$^{13}C_2$]mevalonate, diluted with unlabeled mevalonate, is the starting substrate.[7]

We have examined by ^{13}C NMR two specimens of cholesterol, one biosynthesized from [5-^{13}C]- and the other from [3′,4-$^{13}C_2$]mevalonate. The spectra obtained confirmed the expected distribution of excess ^{13}C in cholesterol and provided an independent check on the assignments of the ^{13}C resonances in cholesterol as well as values of certain ^{13}C–^{13}C coupling constants.

The assignments of the resonances in the ^{13}C NMR spectrum of cholesterol were originally made by Reich et al.[9] and confirmed by Mantsch and Smith,[10] but the resonances of C-12, C-16, and C-20 were only tentatively assigned. Subsequent work[11,12] showed that the original assignments of the resonances of C-12 and C-16 should be reversed.[13] The ^{13}C NMR spectra of biosynthetic [^{13}C]cholesterols provided an independent confirmation of the revised assignments.

Experimental Section

[^{13}C]Cholesterols. Two specimens of [^{13}C]cholesterol were obtained by biosynthesis in vivo in 14-day-old rats (about 20-g body weight) injected over 2 days subcutaneously with four nearly equally spaced doses of [^{13}C]mevalonates (see below), 1 μmol/g of body weight at each injection. The animals were killed 16 h after the last dose and the cholesterol was prepared from the carcasses and organs (excepting

the stomach, brain, and spinal cord).[14] The tissues were first hydrolyzed with 40% KOH at 70 °C for 2 h; the hydrolysates were then diluted with ethanol to a concentration of 50% ethanol. The unsaponifiable lipids were extracted with petroleum ether (bp 40-60 °C) and were fractionated into squalene and sterols by chromatography on alumina.[15] Cholesterol was purified by four crystallizations from ethanol. None of the analytical methods used (thin-layer chromatography, gas-liquid chromatography, gas-liquid radiochromatography, mass spectrometry, and NMR) gave any suggestion of the presence of impurities in the specimens analyzed. If such were present they were below the sensitivity of the detecting powers of our instruments.

The specimen to be referred to as [$^{13}C_6$]cholesterol came from three animals injected with (*RS*)-[5-^{13}C,5-^{14}C]mevalonate (specific activity 0.18 Ci/mol); 45 mg was available for analysis. According to mass spectrometry the specimen consisted of cholesterol with the natural abundance of ^{13}C (98.66%) and of ^{13}C-enriched molecules (1.34%), among which the $^{13}C_5$ molecules (0.425%) and the $^{13}C_6$ molecules (0.642%) predominated. [$^{13}C_3$]Cholesterol was just detectable (0.06%); the abundance of $^{13}C_4$ molecules was 0.213%. A similar pattern of enrichment was found also in squalene. The specimen to be referred to as [$^{13}C_{10}$]cholesterol was obtained from five animals injected with (*RS*)-[3′,4-$^{13}C_2$,5-^{14}C]mevalonate (specific activity 241 μCi/mmol); 107 mg was available for analysis. Mass spectrometry showed the specimen to consist of cholesterol with the natural abundance of ^{13}C (92.7%) and of ^{13}C-enriched molecules (7.6%) among which the di- to hexa-^{13}C molecules predominated, although in spectra of high intensity even [$^{13}C_{10}$]cholesterol was detectable.

[5-^{13}C]Mevalonolactone. This was synthesized from 4,4-dimethoxybutan-2-one and methyl bromo[1-^{13}C]acetate (Merck, Canada) by an improvement[16] of a method described originally for the synthesis of [4-^{14}C]mevalonolactone.[7]

According to mass spectral and ^{13}C NMR analysis 90% of the molecules in the preparation contained ^{13}C at C-5. [5-^{14}C]Mevalonolactone was added to a portion of the ^{13}C-labeled specimen to a specific activity of 180 μCi/mmol. This addition reduced the ^{13}C abundance of the specimen to 88.9%.

[3′,4-$^{13}C_2$]Mevalonolactone.[17] This specimen was obtained from the mother liquor of a preparation, synthesized in 1957 for the study of methyl migration during the cyclization of squalene (squalene 2,3-dioxide) to sterol.[7,18] We added to the dark brown residue (68.5 mg) of this mother liquor 8.9 μmol of [5-^{14}C]mevalonolactone (Schwartz-Mann; specific activity 11.2 Ci/mol) and purified the mixture by liquid-liquid partition chromatography, as described[19] on a Celite-H_2SO_4 column (20 g of Celite + 16 ml of 0.5 N H_2SO_4, stationary phase) eluted with $CHCl_3$ equilibrated with 0.5 N H_2SO_4. All colored material was eluted from the column in the first 40 ml of effluent which contained no ^{14}C. All the radioactive material was eluted from the column between 132 and 228 ml of effluent. These fractions were pooled and the solvent was evaporated off; the residue, a colorless oil, 55 mg, was assayed (after opening of the lactone ring with KOH) with mevalonate kinase[20] and shown to contain 208.5 μmol of (*R*)-mevalonate. Radioactive analysis of the lactone in a Packard Tri-Carb scintillation spectrometer gave a specific activity of 241 μCi/mmol. Mass spectrometric analysis of the purified lactone showed it to contain 24.2% of [3′,4-$^{13}C_2$]- and 23.7% each of [3′-$^{13}C_1$]- and [4-$^{13}C_1$]mevalonolactone and 28.4% of nonisotopic species. These values are very close to those reported previously[7] and which were calculated from the ^{13}C content of the [2-^{13}C]acetic acid used for the synthesis of the lactone.

Spectrometry. Mass spectra were taken with an MS-902 mass spectrometer (A.E.I., Ltd., Manchester, England). For determination of isotopic abundances in the various substances, the molecular ion regions were scanned slowly with wide collector slits so as to obtain broad flat tops for each recorded ion beam. The isotopic abundances were calculated from ten scans.

The ^{13}C NMR spectra were measured on a Varian CFT-20 spectrometer at a frequency of 20 MHz. The samples were examined in $CDCl_3$ solutions in 8-mm tubes. The spectra are Fourier transforms carried out on accumulations of 50 000-75 000 transients. Time delays of from 1 to 2 s and 90 °C pulses were used.

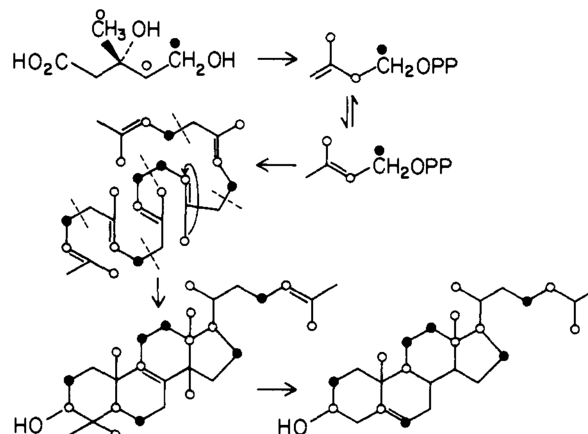
Cholesterol exhibited the following chemical shifts in chloroform solution containing tetramethylsilane as a reference (shifts are given in parts per million downfield from the reference line): C-1, 37.6; C-2, 32.0; C-3, 72.1; C-4, 42.6; C-5, 141.1; C-6, 122.0; C-7, 32.2; C-8, 32.2; C-9, 50.5; C-10, 36.8; C-11, 21.4; C-12, 40.1; C-13, 42.6; C-14, 57.1; C-15, 24.6; C-16, 28.5; C-17, 56.5; C-18, 12.0; C-19, 19.6; C-20, 36.1; C-21, 18.9; C-22, 36.5; C-23, 24.2; C-24, 39.8; C-25, 28.3; C-26, 22.7; C-27, 22.9. These shifts are close to those reported for cholesterol in other solvents.[11,13] The intensities of the resonances of the labeled cholesterols were compared with those of unlabeled cholesterol obtained under the same instrumental conditions and at concentrations identical with those used in the analysis of the labeled specimens.

Results and Discussion

It is pertinent to examine the nature and extent of the labeling expected in the two cholesterol samples. Since the steady-state concentration of mevalonate in the rats should be low,[21] the injected labeled mevalonates were not expected to be diluted much by endogenous substrate particularly as administered mevalonate, in doses that were given (1 μmol/g at each injection), inhibits endogenous synthesis of this intermediate.[22] Thus, the only significant dilution of the cholesterol newly synthesized from the [^{13}c0mevalonates was probably by the preexisting cholesterol possessing ^{13}C only in natural abundance. Although the excess ^{13}C content of the isolated cholesterol samples was relatively low (see Experimental Section), ^{13}C-^{13}C couplings could be observed because of the high ^{13}C content of the newly synthesized fraction of the total cholesterol sample.

The route of incorporation of ^{13}C from the mevalonates is shown in Scheme I. The cholesterol obtained from [5-^{13}C]-

Scheme I. Overall Path of Cholesterol Biosynthesis from Mevalonate[a]



[a] Positions in squalene, lanosterol, and cholesterol derived from C-5 of mevalonate are marked with closed circles and those from C-3′ and -4 are marked with open circles. The broken lines in the formula of squalene show the isoprenoid units derived from individual mevalonate molecules.

mevalonate should be labeled at six positions, namely, 2, 6, 11, 12, 16, and 23; this compound will be referred to as [$^{13}C_6$]-cholesterol. The cholesterol obtained from [3′,4-$^{13}C_2$]mevalonate should be labeled at ten positions: 3, 5, 9, 13, 17, 18, 19, 21, 24, and 27; it will be referred to as [$^{13}C_{10}$]cholesterol. Mass spectrometry on [$^{13}C_6$]cholesterol showed that only 1.34% of the cholesterol molecules contained excess ^{13}C, but 48% of these molecules had six ^{13}C labels and 32% had five (cf. Experimental Section). Therefore, most (ca. 80%) of the molecules should have ^{13}C labels at both C-11 and C-12, and one-bond ^{13}C-^{13}C spin coupling should be easily detected in the ^{13}C NMR spectrum. Triply labeled molecules, e.g., with ^{13}C at C-11, C-12, and C-16, should occur in substantial amounts (ca. 70%) and should give rise to a three-bond (vicinal) ^{13}C-^{13}C spin coupling, as well as to the one-bond coupling.

The proton-decoupled ^{13}C NMR spectrum of [$^{13}C_6$]cholesterol (see Figure 1 for the high-field region of the spectrum) shows that carbons 2, 11, 12, 16, and 23 contain about twice as much ^{13}C, per position, as do the other carbons, as expected. This was also true for C-6 whose resonance is further downfield

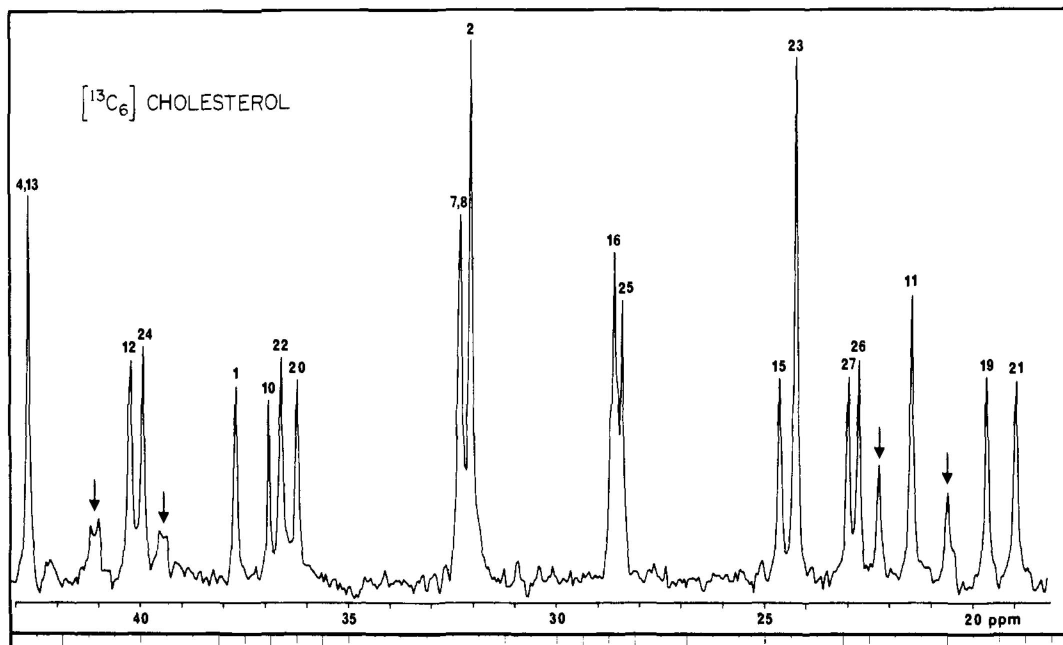


Figure 1. Upfield portion of the proton-decoupled 20-MHz ^{13}C NMR spectrum of [$^{13}C_6$]cholesterol.

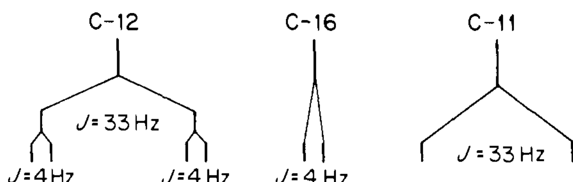


Figure 2. Diagrammatic representation of the C-11, C-12, and C-16 resonances in the spectrum of [$^{13}C_6$]cholesterol in those molecules that are labeled at each of these three positions.

from those shown in Figure 1. The labeling at C-2, C-6, and C-23 leads to a simple increase in the intensities of these resonances. The situation is more complex at the other labeled positions, because of the presence of spin coupling. The C-11 resonance is seen as a single line in the center of a doublet, indicated by arrows in Figure 1. The single line is slightly more intense than would be expected from the natural abundance of ^{13}C, and this reflects the small proportion of labeled molecules which have a ^{13}C at C-11 and a ^{12}C at C-12. The doublet, whose total intensity indicates about 1% ^{13}C labeling at C-11, arises from molecules having ^{13}C atoms at both positions 11 and 12. It is not expected that C-11 will show observable couplings to any other labeled atoms. The splitting (33 Hz) of the C-11 doublet is equal to the coupling constant ($^1J_{11,12}$) and its value is typical of directly bonded sp^3 carbons.[23] The ^{13}C signal of C-12 is similar to that of C-11 in showing a central line flanked by two satellites ($J \approx 33$ Hz), shown by arrows in Figure 1. In this case, each satellite is not a singlet but a narrowly spaced doublet ($^3J_{12,16} \approx 4$ Hz). The extra splitting comes from a vicinal coupling to C-16 and can only arise from triply labeled molecules. Doubly labeled molecules at C-11 and -12 alone would not exhibit the extra coupling and must be present only to a small extent. Finally, the resonance of C-16, upon careful examination, can be seen to be a single central line whose lower part is abnormally wide because it actually contains an unresolved doublet ($J \approx 4$ Hz) from coupling to C-12. The value of $^3J_{12,16}$ is that expected for a vicinal coupling between two sp^3-hybridized ^{13}C atoms bonded in an arrangement having a dihedral angle close to 180°,[26] as is indeed the case in cholesterol. Gauche arrangements, i.e., dihedral angles of ±60°, lead to very small coupling constants (1-2 Hz).[26]

A schematic drawing of the resonances of cholesterol triply labeled at C-11, C-12, and C-16 is shown in Figure 2. The vicinal coupling ($^3J_{12,16}$) should be equally evident in the resonances of C-12 and C-16 for cholesterol that is labeled only at C-12 and C-16 or is completely labeled at positions 11, 12, and 16, as can be seen from Figure 2. The presence of a large amount of cholesterol with the natural abundance of ^{13}C (i.e., effectively singly labeled molecules) makes the observation, and especially the measurement of $^3J_{12,16}$, difficult when the resonance of C-16 is examined. For C-12, however, the large one-bond coupling to C-11 shifts the doublet resulting from $^3J_{12,16}$ away from the large central peak of cholesterol containing ^{13}C in natural abundance and thus allows a very easy measurement of the vicinal coupling constant.

The assignments of the C-12 and C-16 resonances given in Figure 1 follow unambiguously from the coupling pattern of C-11, C-12, and C-16 and are in agreement with the revisions recently made.[11,12]

Mass spectrometry on the [$^{13}C_{10}$]cholesterol, which was derived from [3′,4-$^{13}C_2$]mevalonate, showed that 7.7% of the molecules in it were labeled and that the main labeled species contained two to six atoms of ^{13}C (see Experimental Section). The relatively small number of ^{13}C atoms in this specimen of cholesterol is accountable by the low isotope content of the [3′,4-$^{13}C_2$]mevalonate[24] (cf. also Experimental Section).

From the mass spectrometric data on the [$^{13}C_{10}$]cholesterol, and its mevalonate precursor, it is possible to calculate that the probability of any two positions being labeled in the same molecule of cholesterol is about 24% if the two labeled positions originated from the same mevalonate molecule, but only about 6% if they came from different mevalonate molecules, provided that the injected mevalonate was not diluted significantly by endogenous substrate. This argument applies specifically to the following pairs of carbon atoms of cholesterol: (C-5 + C-19); (C-17 + C-21); (C-27 + C-24); and (C-13 + C-18). The last pair is of particular interest as it was shown[7] that C-18 arrives at C-13 by a 1:2 methyl migration within a specific isoprenoid unit of squalene. Thus when this isoprenoid unit is derived from the doubly labeled component of the [3′,4-$^{13}C_2$]mevalonate specimen, both C-13 and C-18 should be labeled and give an observable AX quartet. If C-18 had arrived at C-13 by a 1:3 migration from position 8, rather than from

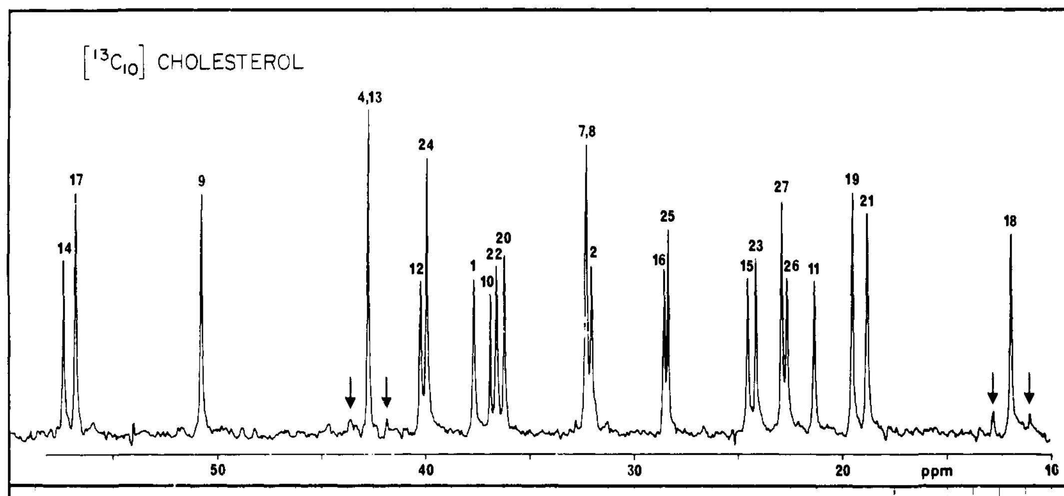


Figure 3. Upfield portion of the proton-decoupled 20-MHz ^{13}C NMR spectrum of [$^{13}C_{10}$]cholesterol.

position 14 by 1:2 migration, the AX quartet would be too weak to be observed (see Scheme I).

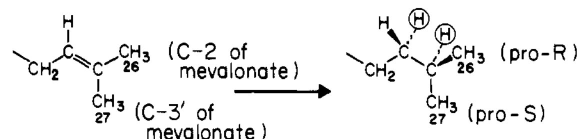
Figure 3 shows the high-field region of the proton-decoupled ^{13}C NMR spectrum of [$^{13}C_{10}$]cholesterol. The resonances of C-3, C-5, and C-6, although observed, are not shown in this expanded spectrum. From peak intensities, ^{13}C labels were found definitely at positions 3, 5, 9, 17, 18, 19, 21, 24, and 27. Position 13 is also almost certainly labeled, but an overlap of the resonances of C-13 and C-4 prevents a firm conclusion.[25] All the resonances, except those of C-13, C-17, and C-18, are observed as single lines. Actually, most of the labeled positions, e.g., C-18, should show geminal ^{13}C-^{13}C couplings, but the small abundance of molecules doubly labeled at two specific sites, combined with the small value expected for ^2J,[26] prevents any splitting from being seen at the base of the peaks. For C-13, C-17, and C-18, relatively large one-bond couplings are expected, and, indeed, satellites ($^1J_{13,18} \approx 35$ Hz) are clearly visible on the sides of the central peak of the C-18 resonance. The sum of the intensities of these satellites is clearly in much better agreement with a 1:2, than with a 1:3 methyl-shift mechanism. It needs to be emphasized that a distinction between a 1:2 and 1:3 methyl migration, in respect of the origin of C-18 of cholesterol, could be made only by the use of a specimen of [3′,4-$^{13}C_2$]mevalonate that was diluted with unlabeled mevalonate. In the ideal experiment a specimen of pure [3′,4-$^{13}C_2$]mevalonate, i.e., fully labeled at both positions, diluted with an equal amount of unlabeled mevalonate, should be used.[7] The statistical arguments presented by Cornforth et al.[7] apply equally well to methods of analysis by chemical degradation or by ^{13}C NMR. If pure [3′,4-$^{13}C_2$]mevalonate had been used in the experiment, there would have been an equal chance of C-13 and C-18 being equally labeled with ^{13}C whether C-18 arrived at C-13 by a 1:3 migration from C-8 or by a 1:2 migration from C-14.

Satellites are also visible on the sides of the C-13 peak but are less intense than those around the C-18 peak. Although the signal-to-noise ratios for the satellites are poor, it is probable that the low intensity arises in part because C-13 is also coupled to C-17. The probability of ^{13}C atoms being at both 13 and 17 is not large, as the labels arise from different mevalonate molecules, but the coupling should reduce the intensities of the C-13 satellites marked with arrows in Figure 3 by 25%. The lost intensity should appear as satellites to the satellites, but the multiple splittings lead to unobservable peaks.

In the case of C-17, a very low intensity satellite can barely be seen on the high-field region of the central resonance of this carbon; the low-field satellite is unfortunately obscured by the C-14 peak. The satellite intensity is of course expected to be very low because the splitting is caused by $^1J_{13,17}$ and, as discussed previously, the labels at C-13 and -17 originate from different mevalonate residues. From the mass spectrometric data, the satellites of C-17 should be four times weaker than those of C-18, and this is certainly not inconsistent with the observed ^{13}C spectrum.

Comparison of the spectra of the [$^{13}C_6$]- and [$^{13}C_{10}$]cholesterol shows also that of the two positions marked 26 and 27 the latter was enriched with ^{13}C in the specimen of [$^{13}C_{10}$] cholesterol. C-26 and C-27 of cholesterol correspond to the terminal methyl groups at one end of squalene and are derived ultimately from the methyl groups of 3,3-dimethylallyl pyrophosphate. These methyl groups have different origins: the (*Z*)-methyl group is derived from C-3′ of mevalonate and the (*E*)-methyl group is furnished by C-2 of mevalonate (see Scheme I). In the side chain of lanosterol, or desmosterol, which contains the 24(25) double bond, C-26 (*E*) and C-27 (*Z*) correspond, respectively, to the (*E*)- and (*Z*)-methyl groups of 3,3-dimethylallyl pyrophosphate (Scheme II). In the

Scheme II. Stereochemistry of the Reduction of the 24(25) Double Bond in Lanosterol or Desmosterol[a]



[a] The new hydrogen atoms introduced are marked with circles.

side chain of cholesterol the origins of C-26 and C-27 could not be distinguished unless the stereochemistry of the reduction of the 24(25) double bond were known. Caspi and his colleagues[27] have deduced that in the reduction of the 24(25) double bond both hydrogens were added to the *re* face of the double bond, equivalent to the cis addition of the hydride ion to C-25 from a pyridine nucleotide and of a proton to C-24. It follows, since C-25 in cholesterol is a prochiral center, that C-26 and C-27 of lanosterol (or of desmosterol) become, respectively, the pro-*R* (C-26) and the pro-*S* (C-27) methyl groups in the side chain of cholesterol (Scheme II). Thus we can firmly assign the resonance at 22.9 ppm downfield from tetramethylsilane to the pro-*S* (C-27) methyl group of cholesterol. The assignments of the resonances of C-26 and C-27 have been made, so far, arbitrarily, but they happen, accidentally, to be correct. The magnetic nonequivalence of C-26 and -27 of cholesterol is shared by their enzymic inequality

also. For example, *Mycobacterium smegmatis*, which converts cholesterol into 26-hydroxycholest-4-en-3-one, hydroxylates specifically the methyl group on the side chain of cholesterol that is derived from C-2 of mevalonate and confers the *S* configuration on C-25.[27] It may be recalled that the enzymic nonequivalence of the terminal methyl groups of squalene was inferred at the time of the first chemical degradation of squalene biosynthesized from [2-^{14}C]mevalonate,[3b] although the chemical degradation could not, of course, distinguish between these methyl groups, just as in the degradation of cholesterol biosynthesized from [2-^{14}C]mevalonate it could not be decided whether C-26 or -27 (or both) carried the label.[5]

We propose, for purposes of nomenclature, that the pro-(*R*)-methyl group at C-25 and derived from C-2 of mevalonate be numbered 26 and the pro-(*S*)-methyl group, derived from C-3′ of mevalonate, be numbered 27.

In summary, the combined mass spectrometric and ^{13}C NMR data on two different ^{13}C-labeled cholesterols obtained by biosynthesis from mevalonate reveal in a simple way the incorporation pattern, including the interesting 1:2 methyl shift, and confirm the revised assignments of the ^{13}C resonances in cholesterol. The easy detection of ^{13}C–^{13}C spin couplings despite the low overall ^{13}C enrichment of the cholesterol obtained in the experiments with a monolabeled precursor is completely understandable, since the highly labeled [5-^{13}C]mevalonate gave a high proportion of molecules labeled at C-11, -12, and -16 in the newly synthesized cholesterol, even though the percentage of these newly synthesized molecules in the specimen examined was low. Usually, the observation of ^{13}C–^{13}C spin coupling requires that highly enriched dilabeled precursors be used or that a very high incorporation of a monolabeled precursor take place.[28]

Acknowledgments. We thank Dr. Siu-May Wong for the synthesis of the [5-^{13}C]mevalonolactone and Dr. Rita H. Cornforth for the gift of the old specimen of [3′,4-$^{13}C_2$]mevalonolactone. The work was supported in part by U.S. Public Health Service Research Grant HL-12745 to G.P. and Research Grant GP-36504X to F.A.L.A. from the National Science Foundation. The Varian CFT-20 NMR spectrometer was purchased from funds of the National Science Foundation awarded to the Chemistry Department, UCLA.

References and Notes

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(2) (a) J. Würsch, R. L. Huang, and K. Bloch, *J. Biol. Chem.*, **195**, 439 (1952); (b) J. W. Cornforth, G. D. Hunter, and G. Popják, *Biochem. J.*, **54**, 590, 597 (1953); (c) J. W. Cornforth, I. Gore, and G. Popjak, *ibid.*, **65**, 94 (1957); (d) K. Bloch, *Helv. Chim. Acta*, **36**, 1611 (1953); (e) W. G. Dauben and K. H. Takemura, *J. Am. Chem. Soc.*, **75**, 6302 (1953).
(3) (a) J. W. Cornforth and G. Popják, *Biochem. J.*, **58**, 403 (1954); (b) J. W. Cornforth, R. H. Cornforth, G. Popják, and I. Youhotsky Gore, *ibid.*, **66**, 10P (1957); **69**, 146 (1958); (c) F. Dituri, S. Gurin, and J. L. Rabinowitz, *J. Am. Chem. Soc.*, **79**, 2650 (1957).
(4) P. A. Tavormina, M. H. Gibbs, and J. W. Huff, *J. Am. Chem. Soc.*, **78**, 4498 (1956).
(5) O. Isler, R. Rüegg, J. Würsch, K. I. Gey, and A. Pletscher, *Helv. Chim. Acta*, **40**, 2369 (1957).
(6) (a) R. B. Woodward and K. Bloch, *J. Am. Chem. Soc.*, **75**, 2023 (1953); (b) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).
(7) J. W. Cornforth, R. H. Cornforth, A. Pelter, M. G. Horning, and G. Popják, *Tetrahedron*, **5**, 311 (1959).
(8) R. K. Maudgal, T. T. Tchen, and K. Bloch, *J. Am. Chem. Soc.*, **80**, 2589 (1958).
(9) H. J. Reich, M. Jautelet, M. T. Messe, F. J. Weigert, and J. D. Roberts, *J. Am. Chem. Soc.*, **91**, 7445 (1969).
(10) H. H. Mantsch and I. C. P. Smith, *Can. J. Chem.*, **51**, 1384 (1973).
(11) W. B. Smith, D. L. Deavenport, J. A. Swanzy, and G. A. Pate, *J. Magn. Reson.*, **12**, 15 (1973).
(12) J. W. ApSimon, H. Beierbeck, and J. K. Saunders, *Can. J. Chem.*, **51**, 3874 (1973).
(13) The assignments of C-12 and C-16 in cholesterol (L. F. Johnson and W. C. Jankowski, "Carbon-13 NMR Spectra", Wiley-Interscience, New York, N.Y., 1972, p 494) should also be reversed.
(14) The stomach was rejected because in suckling rats it is full of mother's milk; the brain and spinal cord were also excluded because mevalonate appears not to pass the blood–brain barrier and because brain cholesterol even in the young developing rat injected with either [5-^{13}C]- or [3′,4-$^{13}C_2$]mevalonate contains a barely detectable level of excess ^{13}C (G. Popják, unpublished observations).
(15) R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 129 (1953).
(16) R. H. Cornforth and G. Popják, *Methods Enzymol.*, **15**, 359 (1969).
(17) This compound is named [4,6-$^{13}C_2$]mevalonate in the present Chemical Abstracts nomenclature but has been named originally [3′,4-$^{13}C_2$]mevalonate in the biochemical literature.
(18) We thank Dr. Rita H. Cornforth, who preserved the mother liquor of the preparation from which the [$^{13}C_2$]mevalonolactone used in the first study was crystallized, for giving the residues to us. The purification of the mevalonolactone from this old specimen and the experiment described here were carried out in 1972 attesting to a remarkable stability of mevalonolactone.
(19) C. Donninger and G. Popják, *Proc. R. Soc., London, Ser. B*, **163**, 465 (1966).
(20) H. R. Levy and G. Popják, *Biochem. J.*, **75**, 417 (1960).
(21) The physiological concentration of (*R*)-mevalonate in rat blood is about 250 pmol/ml of plasma: G. Popják and G. Boehm, unpublished results.
(22) P. Edwards and A. M. Fogelman, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **35**, 1472 (1976).
(23) P. D. Ellis and R. Ditchfield in "Topics in Carbon-13 NMR Spectroscopy", Vol. 2, Wiley-Interscience, New York, n.Y. 1976.
(24) The [2-^{13}C]acetyl chloride used in the synthesis of the 3-oxobutan-1-ol, the starting material for the synthesis of the mevalonolactone, contained only 52.6% ^{13}C-labeled molecules and the [$^{13}C_2$]oxobutanol itself was diluted further with unlabeled substance. See ref 7.
(25) Labeling at C-13 is suggested by the comparison of the ratio of the intensity of the 4,13 resonance line to the sum of the intensities of lines of C-1, -10, -22, and -20 in the spectrum of the [$^{13}C_6$]cholesterol to the similar ratio in the spectrum of the [$^{13}C_{10}$]cholesterol. The former gave a value of 0.48 and the latter a value of 0.51. None of these positions should be labeled in the [$^{13}C_6$]cholesterol nor should positions 1, 10, 22, 20, and 4 contain excess ^{13}C in the [$^{13}C_{10}$]cholesterol; only C-13, among the positions listed, should predictably contain label in the latter specimen.
(26) J. L. Marshall, D. E. Miller, S. A. Conn, R. Seiwell, and A. M. Ihrig, *Acc. Chem. Res.*, **7**, 333 (1974); M. Barfield, D. Doddrell, I. Burfitt, and J. M. Grutzner, *J. Am. Chem. Soc.*, **96**, 1241 (1974).
(27) M. G. Kienle, R. K. Varma, L. J. Mulheirn, B. Yagen, and E. Caspi, *J. Am. Chem. Soc.*, **95**, 1996 (1973).
(28) A. G. McInnes, J. A. Walter, J. L. C. Wright, and L. C. Vining in "Topics in Carbon-13 NMR Spectroscopy", Vol. 2, Wiley-Interscience, New York, N.Y., 1976; D. M. Wilson, A. L. Burlingame, T. Cronholm, and J. Sjövall, *Biochem. Biophys. Res. Commun.*, **56**, 828 (1974); D. P. H. Hsieh, J. N. Seiber, C. A. Reece, D. L. Fitzell, S. L. Yang, J. I. Dalezios, G. N. LaMar, D. L. Budd, and E. Motell, *Tetrahedron*, **31**, 661 (1975); T. J. Simpson and J. S. E. Holker, *Tetrahedron Lett.*, 4693 (1975).