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Reduction of Δ^{24} of Lanosterol in the Biosynthesis of Cholesterol by Rat Liver Enzymes. I. The Addition of a 24-Pro-*S* Proton[1]

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Abstract: The side chain of 17α,20,24*R*-$^3H_3$-$^{14}C_5$-cholesterol biosynthesized from 4*R*-(2-^{14}C,4-^3H)-mevalonic acid by rat liver enzymes was cleaved with an adrenal enzyme preparation and the 1,5-$^{14}C_2$-3*R*-$^3H_1$-isocaproic acid encompassing carbons 22–27 of cholesterol was isolated. The $^{14}C_2$,$^3H_1$-isocaproic acid was degraded, without disturbing the stereochemistry of the isotopic hydrogen, to 1*S*-3-^{14}C,1-^3H-isobutyl alcohol. The isobutyl alcohol was oxidized with NAD^+ and yeast alcohol dehydrogenase (YADH) to 3-^{14}C,1-^3H-isobutyraldehyde with complete retention of tritium. Since we proved that NAD^+–YADH oxidation of isobutyl alcohol proceeds with the abstraction of the 1-pro-*R* proton it follows that the isobutyl alcohol has the 1*S* stereochemistry. Hence the ^{14}C,^3H-cholesterol must have the 24*R* stereochemistry. This implies that a 24-pro-*S* proton adds to Δ^{24} of lanosterol in the biosynthesis of cholesterol by rat liver enzymes. It was also demonstrated with the use of samples of 1-$^3H_1$-isobutyl alcohols that no significant isotope effects were detected in the NAD^+–YADH oxidation of the alcohol to isobutyraldehyde by the procedures employed.

The biosynthetic transformation of lanosterol (**1**) to cholesterol (**2**) is a multistep process which involves among other transformations the reduction of the C-24 double bond.[3] In this paper we concern ourselves with the mode of reduction of this double bond in a rat liver enzyme system,[4a,b] and particularly with the stereochemistry of addition of the hydrogen at C-24. At the time that this work was undertaken it was already known[5a] that the biosynthesis of squalene, lanosterol, and cholesterol from exogenously supplied mevalonic acid (MVA) proceeds with the stereospecific retention of, respectively,[5a] six, five, and three 4-pro-*R* protons of MVA. Of the three protons retained in cholesterol the presence of the one at C-17 was proven experimentally.[5a] The other two were thought to be located at C-20[5b] and -24,[4b] which we later confirmed.

The terminal methyls of lanosterol (and of cholesterol (**2**)) differ in that one originates from C-2 and the other from C-3′ of MVA. From the work of Birch, *et al.*,[6a] it was inferred that the geometry at Δ^{24} of lanosterol is that shown in **1** (the heavy dots indicate carbons derived from C-2 of MVA, and the encircled protons indicate hydrogens originating from the 4-pro-*R* position of MVA). Subsequently the hypothesis was confirmed experimentally.[6b]

The sequence of biosynthetic reactions in the transformation of lanosterol to cholesterol is unknown. However, to simplify discussion we will for the present assume that demosterol (**4**) is the immediate precursor of cholesterol. It follows therefore that acquisition by demosterol of two hydrogens at C-24 and -25 will give cholesterol. We will now analyze the possible mechanisms of this reduction from the point of view of events at C-24. The addition of a hydrogen at C-24 can occur either from the front or back of the double bond. In a backside attack the encircled C-24 proton thought to be derived from the 4-pro-*R* position of MVA will be pushed forward as in **5** and **6**. If the overall reduction entails a cis addition of two hydrogens, the resulting cholesterol will have the configuration shown in **5**. On the other hand if the reduction is a trans process the biosynthetic cholesterol will have the configuration as in **6**. By a similar reasoning, should the attack at C-24 occur from the front side, a cis or trans reduction will result in cholesterols **7** and **8**, respectively. Hence, for the determination of the overall mechanism of the reduction of Δ^{24} it is necessary to define the prochirality at C-24[4a] and C-25[4b] of the biosynthetic cholesterol.

We focused first on the events taking place at C-24 which are described in this paper.[4a] Our plan of attack was to biosynthesize cholesterol from MVA stereospecifically labeled with isotopic hydrogen at the 4-pro-*R* position and then to determine the configuration at C-24. The most direct approach would have been to biosynthesize cholesterol from 4*R*-4-$^2H_1$-MVA and

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(2) (a) Postdoctoral Fellow, 1966–1968; (b) Postdoctoral Fellow, 1966–1969.

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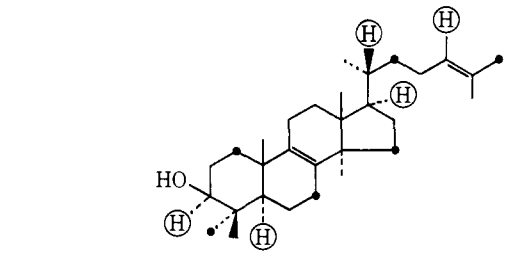
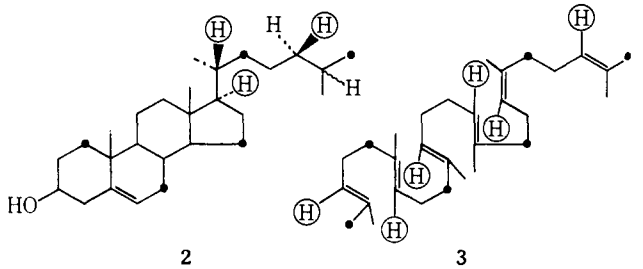


1, • ≡ derived from C-2 of MVA
Ⓗ ≡ derived from 4-pro-*R*-H of MVA



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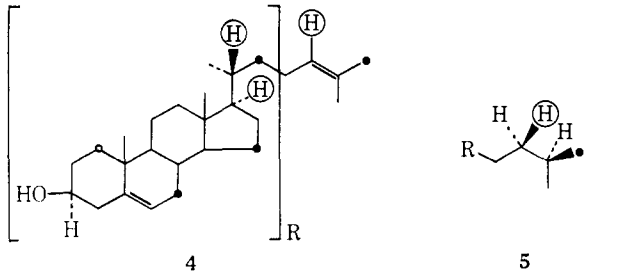

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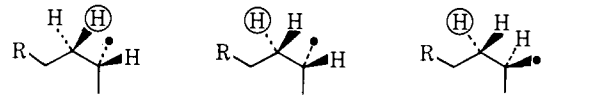

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relate its rotation to authentic 24*R*- and 24*S*-24-$^{2}H_{1}$-cholesterol. Although the biosynthesis of multimilligram amounts of cholesterol labeled with deuterium was a demanding task it could still be accomplished.[7] A more disturbing aspect was our conviction that the rotational increment due to the asymmetry at C-24 induced by deuterium would be obscured by the large contributions emanating from the other asymmetric centers of cholesterol. It was felt that measuring of the rotation of a fragment encompassing carbons C-22–27 (*e.g.*, isocaproic acid) apart from the rest of the molecule was considerably more promising. In fact we have synthesized samples of 3*R*- and 3*S*-3-$^{2}H_{1}$-isocaproic acids and determined their specific rotations.[8] Unfortunately this route proved wholly impractical because the amounts of isocaproic acid required for rotational measurements could not be prepared by the then available method of cholesterol side-chain cleavage with an adrenal enzyme preparation.[9–11] It became obvious that classical methods of configurational assignment could not be used and other more precise microscale procedures would have to be employed. Under the circumstances we changed the approach and concentrated on the use of tritiated mevalonic acid. We planned to biosynthesize cholesterol from 4*R*-(2-^{14}C,-4-^{3}H)-MVA,[4,12] cleave its side chain with an adrenal enzyme preparation,[9] and isolate the 1,5-$^{14}C_{2}$,3-$^{3}H_{1}$-isocaproic acid. The doubly labeled 1,5-$^{14}C_{2}$,3-^{3}H-isocaproic acid would be degraded chemically to 1-^{3}H,-3-^{14}C-isobutyl alcohol without disturbing the configuration of the tritium atom.[13] Ultimately the configuration at C-1 of the isobutyl alcohol would be determined with NAD^{+}– and yeast–alcohol dehydrogenase, which we have proved oxidizes isobutyl alcohol to isobutyraldehyde by abstracting the 1-pro-*R* proton.[13]

Experimental Section

Female albino rats (body weight 180–200 g) of the Sprague-Dawley strain were purchased from Charles River Laboratories, N. Willmington, Mass. Adrenal glands of cows were obtained within 1 hr of slaughtering and were transported to the laboratories on ice. All enzyme preparations were carried out at 0–4°.

ATP, GSH, and glucose 6-phosphate were purchased from Sigma Chemical Company, St. Louis, Mo. NAD^{+}, NADH, $NADP^{+}$, glucose-6-phosphate dehydrogenase, and yeast alcohol dehydrogenase were used as supplied by Calbiochem, Los Angeles, Calif. Organic solutions were dried over anhydrous $Na_{2}SO_{4}$. Melting points were measured on a hot stage and are corrected.

Radioactivity. The dibenzylethylenediamine salt of 2-^{14}C-mevalonic acid was purchased from New England Nuclear Corporation, Boston, Mass. Lithium borotritide was obtained from New England Nuclear Co., Boston, Mass., and was used for the preparation of the diphenylmethylamide of 4*R*-(4-^{3}H)-mevalonic acid.[14a] Radioactive samples were counted[14b] on a Nuclear Chicago Mark I liquid scintillation counter as solutions in a scintillation fluid containing 2,5-diphenyloxazole (4 g) and 1,4-bis-(2-(5-phenyloxazolyl))benzene (0.1 g) in toluene (1 l.). Liquifluor, a 25-fold scintillation fluid concentrate, was purchased from Nuclear Chicago. Certain derivatives (dimedone and acid phthalate) caused small quenching under the conditions used and the results are corrected.

Gas–Liquid Chromatography (Glc). Glc was carried out on an F & M Model 720 gas chromatograph equipped with a thermal conductivity detector. Helium was the carrier gas. For analytical runs we used $^{1}/_{8}$ in. × 6 ft columns of (i) 5% SE-30 on Chromosorb and (ii) 25% TCEP on Chromosorb. A $^{1}/_{2}$ in. × 6 ft column of packing (i) was used for preparative work. Compounds were identified by the peak enhancement technique with authentic samples.

Thin-Layer Chromatography (Tlc). Tlc was carried out on plates coated with 0.75-mm layers of silica gel $HF_{254+366}$ (E. Merck, A. G., Darmstadt, Germany). Compounds were visualized with ultraviolet light of wavelengths 254 or 366 mμ and located for comparison with authentic marker spots. Solvent systems (a) hexane, (b) benzene–hexane (1:3, v/v), (c) benzene, (d) ethyl acetate–hexane (3:7, v/v), and (e) methanol–benzene (1:9, v/v) were used.

Spectroscopy. Infrared spectra were recorded on a Perkin-Elmer Model 237 instrument as liquid films. Uv measurements were made on a Perkin-Elmer 202 spectrophotometer. Mass spectra were recorded on a Varian M-66 instrument. Nmr spectra were recorded at 60 MHz on a Varian DP/DA-60 spectrometer in $CDCl_{3}$ solutions (unless otherwise specified) using tetramethylsilane as an internal standard.

Model Degradation Studies of Nonradioactive Methyl Isocaproate. 4-Methyl-1,1-diphenylpentan-1-ol and 4-Methyl-1,1-diphenylpent-1-ene (10; No ^{14}C and ^{3}H). A solution of methyl isocaproate (9.75 g, 75 mmol) in ether (50 ml) was added to a cooled and stirred solution of phenylmagnesium bromide (187.5 mmol) in ether (150 ml). The mixture was refluxed for 1.0 hr and then 2 *N* hydrochloric acid (200 ml) was added (cooling). The ether solution was separated and the aqueous layer was extracted once with ether. The

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(14) (a) This preparation differed from that of Cornforth, *et al.*,[12] only in that *trans*-5-hydroxy-3-methylpent-3-enoic acid was isolated from the residue left after distillation of acetic acid and the cis lactone by crystallization (ether) at −70°; (b) E. Caspi, J. A. F. Wickramasinghe, and D. O. Lewis, *Biochem. J.*, **108**, 499 (1968).

combined ether extract was washed with a dilute sodium hydroxide solution, dried, and evaporated to furnish the carbinol (oil) (30.2 g), ν_{max}^{film} 3480, 3590 cm^{-1} (medium, –OH), and characteristic aromatic bands. Analysis by glc on column i at 225° indicated that the specimen was contaminated with significant amounts of bromobenzene and biphenyl. No further purification of this material was attempted.

A solution of the crude carbinol in benzene (500 ml) containing *p*-toluenesulfonic acid dihydrate (100 mg) was distilled (3 hr) until 300 ml of benzene was collected. The resulting solution was cooled, washed with a dilute solution of sodium bicarbonate and brine, dried, and evaporated *in vacuo* to furnish an oil (29.5 g), ν_{max}^{film} no hydroxyl absorption. This was distilled under reduced pressure and after an initial forerun the fraction bp 123–126° (0.1 mm) (16.0 g) was collected. Analysis of this fraction by glc at 225° on column i indicated the presence of a major component (R_t 9.2 min) and three minor components of which two were identified as biphenyl (R_t 5.6 min) and the parent carbinol (R_t 13.7 min). The third minor component (R_t 3.2 min) was not identified. A specimen of this material was subjected to preparative glc, the major component was isolated, and it was identified as 4-methyl-1,1-diphenylpent-1-ene (**10**): ir ν_{max}^{film} characteristic aromatic absorptions; mass spectrum *m/e* 236 (M^+), 193 ($M - (CH_3)_2CH$), 154 ($M - C_6H_{10}$).

Ozonization of 4-Methyl-1,1-diphenylpent-1-ene (10) and Isolation of Isovaleraldehyde (11) (No ^{14}C and ^3H). A stream of dry ozonized oxygen was bubbled through a solution of the olefin **10** (1.0 g) in dry methylene chloride[15] (15 ml) at −70°. The ozonization was monitored by tlc (benzene–ethyl acetate (19:1)) of aliquots removed at intervals. When the starting material disappeared (usually 15–20 min), the solution was allowed to warm up to room temperature and was stirred with a mixture of zinc dust (0.5 g) and glacial acetic acid (0.5 ml). After 4.0 hr, a slurry of sodium bicarbonate (1.5 g) in water (0.5 ml) was added and the stirring was continued for 1.0 hr. Then, anhydrous sodium sulfate (25 g) was added and the mixture was filtered. The removed solids were washed with several small portions of methylene chloride. The filtrate and washings were combined and concentrated by distillation through a 2-ft column packed with glass helices (25 ml). Analysis of the concentrate by glc (column i) using the external standard procedure indicated a 77% yield of isovaleraldehyde.

The isovaleraldehyde was separated from the higher boiling products (*e.g.*, benzophenone) in the following manner. The solution was distilled nearly to dryness at atmospheric pressure into a cooled receiver. When no more material distilled over, the flask was removed from the oil bath and cooled, methylene chloride (5–10 ml) was added, and the distillation was repeated. This process was repeated three more times. The distillate (50 ml) was analyzed by glc (columns i and ii) and was found to contain 272 mg (75% yield) of isovaleraldehyde. The colored material remaining in the distillation flask was identified by tlc and by glc (225°, column i) as nearly pure benzophenone.

An aliquot of the methylene chloride solution was treated with solutions of 2,4-dinitrophenylhydrazine sulfate in ethanol and furnished the 2,4-DNP-hydrazone derivative of isovaleraldehyde, mp 118–121° (reported 123°).[16]

Baeyer–Villiger Oxidation of Isovaleraldehyde (11) (No ^{14}C or ^3H). The isovaleraldehyde solution (54 ml, 245 mg of **11**) was dried (Na_2SO_4) and decanted into a flask containing freshly dried disodium hydrogen phosphate (2.5 g). The flask was cooled in ice; then a solution of trifluoroperacetic acid was added dropwise (10 min). The trifluoroperacetic acid was prepared by mixing at ice bath temperature trifluoroacetic anhydride (1.33 g), in dry methylene chloride (5 ml) with 80% hydrogen peroxide (270 mg). The disappearance of the aldehyde was monitored by glc and usually the oxidation reaction was completed within 3–4 hr. The reaction was terminated with a saturated solution of sodium carbonate (5 ml) and the stirring was continued for 1 hr. Then, anhydrous sodium sulfate (40 g) was added, the mixture was filtered through Celite, and the solids were washed with several small portions of methylene chloride. The filtrate was concentrated to a small volume by distillation through a 2-ft column packed with glass helices. Glc analysis (column i at 60°) revealed the presence of two major components which were identified as isobutyl alcohol and isobutyl formate by comparison with authentic samples. A small amount of unreacted isovaleraldehyde and two other unidentified minor impurities were also detected. The isobutyl alcohol and isobutyl formate were separated by preparative glc and were trapped in ether at −70°.

A solution of the formate in ether (5 ml) was added to a suspension of lithium aluminum hydride (150 mg) in ether (10 ml). After stirring for 1.0 hr 1 *N* hydrochloric acid was added dropwise until a filterable precipitate was obtained. The solid was removed by filtration on Celite and washed with a small amount of ether. The filtrate was dried (Na_2SO_4) and concentrated by distillation through a 2-ft column packed with glass helices. The concentrate was combined with the isobutyl alcohol previously isolated by preparative glc and was subjected to a short-path distillation, furnishing isobutyl alcohol (78 mg), bp 110° (bath) (1 atm). The specimen was 98% pure by glc analysis (columns i and ii, 60°). It was further characterized as the 3,5-dinitrobenzoate (ethanol), mp 85–86° (reported: 87°). The melting point was not depressed after admixture with an authentic specimen.

Model Oxidation Experiments of Isobutyl Alcohol-1-^3H with NAD^+ Yeast Alcohol Dehydrogenase. 1-*RS*-Isobutyl Alcohol-1-^3H. To a solution of sodium borotritide (20 mg) in dry diglyme (5 ml) was added isobutyraldehyde (1.0 g) and the solution was stirred overnight at room temperature. The reduction was completed by adding lithium aluminum hydride (200 mg) and stirring for 30 min. The excess hydride was decomposed with a few drops of water. Isobutyl alcohol (5.0 g) was then added and the mixture was fractionally distilled through a short Vigreux column, collecting isobutyl alcohol-1-^3H in the fraction bp 100–107° (1 atm) (4.89 g).

Isobutyraldehyde-1-^3H. To a stirred mixture of lead tetraacetate (29.8 g, 62 mmol) in pyridine (250 ml) was added 1-*RS*-isobutyl alcohol-1-^3H (4.57 g, 61.8 mmol).[17] The solution was stirred until it was clear and yellow (about 24 hr). The flask was then equipped with a 6-in. packed column and the distillate (30 ml), bp < 100°, was collected. To this distillate was added isobutyraldehyde (5.0 g) and the mixture was fractionally distilled, collecting the isobutyraldehyde-1-^3H, bp 62–65° (1 atm) (5.6 g). Redistillation of the material furnished isobutyraldehyde-1-^3H free of traces of pyridine.

A sample of the above aldehyde was converted to the semicarbazone by the methanol–pyridine method. The mixture was evaporated to dryness in a stream of nitrogen and the residual solid was subjected to preparative tlc on silica gel plates (ethyl acetate–benzene (80:20)). Two successive purifications by tlc and crystallizations from benzene–hexane gave plates, mp 121.5–123.0° (corrected) (an authentic sample showed mp 120.5–123.5°). The sample was dried under reduced pressure (53° (5 hr)) and recrystallized to constant specific activity (1.98×10^{11} dpm/mol). A sublimed sample showed the same specific activity.

1-*RS*-Isobutyl Alcohol-1-^3H. The isobutyraldehyde-1-^3H prepared in the previous experiment (600 mg) was stirred with lithium aluminum hydride (200 mg) in ether (20 ml) for 1.5 hr. The excess hydride was destroyed by the careful addition of a few drops of water followed by a few drops of concentrated hydrochloric acid. The ether layer was separated and dried (Na_2SO_4), and the solvent was removed by distillation through a 1-ft packed column. The concentrate was distilled through a shorter column to furnish 1-*RS*-isobutyl alcohol-1-$^3H_1$, bp 115–118° (bath) (yield, 520 mg).

The 1-*RS*-isobutyl alcohol-1-$^3H_1$ (1 mmol, 73 mg) was refluxed for 4 hr with pyridine (5 ml) and phthalic anhydride (1.5 mmol; 222 mg). The mixture was cooled, diluted with water (50 ml), and extracted with ether (three 30-ml portions). The ether extracts were then combined and the acidic material was extracted with 1 *N* sodium bicarbonate solution. The combined alkaline extracts were washed once with ether, acidified with concentrated hydrochloric acid, and extracted with ether (three 20-ml portions). The ether extracts were combined, washed with a dilute sodium chloride solution, and dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residual material was dissolved in ligroin (90–120°) and refluxed briefly, and the insoluble material (phthalic acid) was removed by filtration. The filtrate on cooling deposited crystals of the acid phthalate, mp 65–66°. The material was recrystallized (ligroin) and dried under reduced pressure (80° (2 hr)) to constant specific activity (1.98×10^{11} dpm/mol).

1-*S*-Isobutyl Alcohol-1-^3H. The isobutyraldehyde-1-^3H (1.0 g) prepared previously (specific activity, 1.98×10^{11} dpm/mol) was

(15) Because subsequent operations involved manipulations of products in methylene chloride the solvent was carefully purified prior to use to furnish a specimen free of higher boiling impurities.

(16) The physical constants of standard compounds and their derivatives are quoted from "Tables of Identification of Organic Compounds," Chemical Rubber Publishing Co., Cleveland, Ohio, 1960.

(17) R. E. Partch, *J. Org. Chem.*, **30**, 2498 (1965); *Tetrahedron Lett.*, 3071 (1964).

diluted with cold isobutyraldehyde (5.0 g). This diluted sample was used for the yeast reduction described below. A sample of the diluted aldehyde was converted to the semicarbazone which was purified by tlc and crystallized from ethyl acetate–hexane to constant specific activity (2.99×10^{10} dpm/mol).

To a stirred and actively fermenting mixture of glucose (450 g), water (2.91), and bakers yeast (National Corporation) (450 g) at 37° was added a solution of the isobutyraldehyde-1-^3H (4.0 g) in ethanol (10 ml). The stirring was continued overnight. The mixture was then steam distilled, and 2.0 l. of the distillate was collected. The distillate was saturated with salt and was continuously extracted with ether. After 12 hr the extract was removed, and the extraction was then continued with a fresh batch of ether. The two extracts were combined and concentrated through a 3-ft column packed with glass helices. The concentrate was dried (Na_2SO_4) and was fractionated through a 1-ft packed column. The fractions rich in 1*S*-isobutyl alcohol-1-^3H (glc) were collected and combined.

This material was purified three times by preparative glc on an 8-ft column of 20% TCEP on Chromosorb, and finally by distillation to furnish (+)-1*S*-isobutyl alcohol-1-^3H (1.8 g), bp 105–106° (1 atm).

A portion of the alcohol was converted to the acid phthalate by the procedure previously described. The acid phthalate was crystallized and dried *in vacuo* (80° (1 hr)) to constant specific activity (3.60×10^{10} dpm/mol).

Oxidation of 1*RS*-Isobutyl Alcohol-1-^3H with NAD$^+$ and YADH. The oxidation medium was prepared by mixing 0.25 *M* glycine–sodium hydroxide buffer (35 ml) of pH 9.8 with NAD$^+$ (253 μmol, 194 mg, 87% β-DPN) and crystalline yeast alcohol dehydrogenase (YADH) (10 mg) (100%, specific activity 30 μ/mg). An aliquot of this solution (0.5 ml) diluted to 5 ml with the buffer served as blank for the uv estimation. The oxidation was started by adding 1*RS*-isobutyl alcohol-1-^3H (253 μmol, 18.7 mg) of specific activity, 1.98×10^{11} dpm/mol. The progress of the oxidation was followed by measuring the optical density at 340 mμ of appropriately diluted aliquots. When an optical density of 21.0 was reached (20 hr) corresponding to the formation of 118 μmol of NAD$^+$ (47% oxidation), the pH of the medium was brought to 6.5–6.7 by adding the *required* amount of dilute acetic acid.[18] A warm solution of dimedone (100 mg) in water (15 ml) was then added, the solution was heated on a water bath for 15–20 min, and then cooled for 5 hr. The precipitated solids were isolated by filtration. The solids were then treated with hot ethanol and the insoluble material was removed by filtration. The derivative was precipitated from the filtrate by adding water and isolated by centrifugation. This process was repeated once again, and the derivative was dried *in vacuo* at 56° (2 hr) before counting; specific activity, 1.07×10^{11} dpm/mol. A duplicate experiment gave a value of 1.03×10^{11} dpm/mol.

A control experiment was carried out to evaluate the loss of tritium from the aldehyde under the conditions used for the oxidation and the subsequent conversion to the dimedone derivate. The oxidation medium was prepared as above and to this isobutyraldehyde-1-^3H (specific activity of semicarbazone, 1.98×10^{11} dpm/mol) was added. After 20 hr at 25°, the aldehyde was treated with dimedone and the derivative was isolated and purified as before. It showed a specific activity of 2.2×10^{11} dpm/mol.

In another experiment, the aldehyde was stored in distilled water (pH 6.6) and after an analogous work-up, it gave a derivative, with a specific activity of 1.99×10^{11} dpm/mol.

Oxidation of 1*S*-Isobutyl Alcohol-1-^3H with NAD$^+$–YADH. The oxidation of the 1*S*-alcohol-1-^3H (specific activity of acid phthalate, 3.7×10^{10} dpm/mol) was carried out exactly as described above for the racemic alcohol. The dimedone derivative of the aldehyde was isolated in an identical manner and showed a specific activity of 3.4×10^{10} dpm/mol. In another experiment a specific activity of 3.7×10^{10} dpm/mol was obtained.

Oxidation of 1*RS*-Isobutyl Alcohol-1-^3H to Isobutyric Acid. The (±)-isobutyl alcohol-1-^3H (specific activity of acid phthalate, 1.98×10^{11} dpm) (50.4 mg) was mixed with nonradioactive isobutyl alcohol (249.7 mg) in acetone (10 ml). Jones' reagent was then slowly added until the yellow color due to excess reagent persisted for 20 min. Water (50 ml) was then added and the solution was extracted with five 20-ml portions of ether. The ether extracts were then combined and washed with several small portions of a dilute sodium bicarbonate solution. The alkaline extracts were combined, acidified with concentrated hydrochloric acid, saturated with salt, and extracted with ether (four 20-ml portions). The ether extract was washed with saturated brine and dried (Na_2SO_4). The solvent was then evaporated and the residual material was distilled. A middle cut, bp 170° (1 atm), of the isobutyric acid (ir) showed a specific activity of 1.69×10^8 dpm/mol. The acid phthalate of the parent isobutyl alcohol-1-$^3H_1$ had a specific activity of 3.98×10^{10} dpm/mol. (Hence, a maximum of only 0.41% ^{3}H is present on the methine carbon.)

Isolation of NADT from Oxidation of (±)-1*RS*-Isobutyl Alcohol-1-^3H with NAD$^+$ and YADH. The experiment described above for the oxidation of the racemic alcohol was repeated in 25 ml of buffer. A control experiment was also carried out in which the enzyme was inactivated by heating for 3 min in boiling water before the addition of the alcohol. After 20 hr, the uv of an appropriately diluted solution showed that 113 μmol of NADH had been produced. The pH of the solution was then adjusted to 8.2–8.4 by the careful addition of 2 *N* hydrochloric acid (pH meter). This was followed by the addition of a 25% solution of barium acetate (1.5 ml) and ethanol (15 ml). The solid that separated was removed by centrifugation, the clear supernatant liquid was diluted with 95% ethanol (125 ml), and the solution was cooled for 2 hr at −16°. The solid that separated was isolated by centrifugation and was washed twice with ethanol (15 ml) (centrifugation). It was then redissolved in water (15–20 ml) and a solution of Na_2SO_4 (140 mg) in water (1.0 ml) was added. The precipitated $BaSO_4$ was removed by centrifugation and the clear supernatant (which contained the sodium salt of NADH) was made up to 25 ml.

The control experiment was also processed in an identical manner. A 20-fold diluted solution from this control experiment did not show an appreciable absorption at 340 mμ in the uv when run against a similarly diluted buffer as blank. A similarly diluted experimental solution showed an optical density of 1.34 at 340 mμ. This corresponds to the presence of 108 μmol of NADH in the total 25 ml of the original solution.

Aliquots (0.05 ml) of the control and the experimental solutions were counted in "POPOP" containing ethanol. Allowances were made for the appreciable quenching (77.4%) of tritium by ethanol and NADH while calculating the results. The total 25 ml of the experimental solution was thus found to contain 108 μmol of NAD^{3}H with a specific activity of 2.26×10^{10} dpm/mol.

Biosynthetic Studies. 4*R*-(2-^{14}C,4-$^3H_1$)-MVA. The diphenylmethylamide of 4*R*-(4-^3H)-mevalonic acid was hydrolyzed with aqueous 2.5 *N* NaOH.[12] The amine and unhydrolyzed material were extracted with ethyl acetate. The aqueous solution was acidified to pH 6 and then excess *N*,*N*′-dibenzylethylenediamine was added. The excess amine was extracted with ether. To this solution was added the dibenzylethylenediamine salt of 2-^{14}C-MVA (100 μCi). An aliquot of the solution was removed and mixed with nonradioactive MVA and the diphenylmethylamide prepared.[12] This was crystallized (ethyl acetate–hexane, 7:3, v/v) to constant specific activity and ^{3}H:^{14}C ratio (see Table I). This ^{3}H:^{14}C ratio was used as a standard for later calculations.

Table I. ^{14}C-Specific Activity and ^{3}H:^{14}C Ratio of Diphenylmethylamide of 4*R*-(2-^{14}C,4-^3H)-Mevalonic Acid

Crystalln[a]	^{14}C specific activity, dpm/mg	^{3}H:^{14}C ratio
1	4,580	7.98
2	4,660	8.08
3	4,630	8.12
4	4,680	8.03

[a] Crystallized from ethyl acetate–hexane (7:3, v/v).

Biosynthesis of Cholesterol from 4*R*-(2-^{14}C,4-^3H)-MVA. The livers (46 g) from six rats were homogenized by the method of Bucher and McGarrahan[19] in 0.1 *M* phosphate buffer (115 ml, pH 7.4) containing nicotinamide (0.03 *M*) and $MgCl_2$ (0.004 *M*). The homogenate was centrifuged at 700*g* for 25 min and, after removal of a fatty layer by aspiration, the supernatant was decanted and recentrifuged at 12,500*g* for 25 min. A fatty layer was again removed and the supernatant was recentrifuged at 12,500*g*

(18) In experiments with the biosynthetic isovaleraldehyde (**10**), when excess acetic acid was added, the aldehyde derivative showed a significantly lower ^{3}H:^{14}C ratio.

(19) N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.*, **222**, 1 (1956).

for 25 min. Seven 1-ml portions of the resulting $S_{12.5}$ were added to six 50-ml Erlenmeyer flasks, each containing a solution of the dibenzylethylenediamine salt of *4R*-(2-^{14}C,4-^{3}H)-MVA (*ca.* 17.4 μCi of ^{14}C) in water (0.5 ml). The mixtures were incubated aerobically at 37° for 3 hr. After 1 and 2 hr there was added to each flask a solution of NADH (2.5 mg), ATP (7.5 mg), NAD^+ (2.5 mg), ascorbic acid (7.5 mg), $NADP^+$ (2.0 mg), glucose 6-phosphate (32 mg), and glucose-6-phosphate dehydrogenase (5 enzyme units) in 0.025 *M* phosphate buffer (1.5 ml, pH 7.4) containing $MgCl_2$ (0.004 *M*). After 3 hr the contents of the flasks was combined and refluxed for 3 hr with a 10% solution of KOH in aqueous methanol (1:1, v/v, 38 ml). The methanol was removed under a stream of nitrogen and the residue was extracted with hexane (five 250-ml portions). The extract was dried and evaporated under reduced pressure. The residue (29 μCi of ^{14}C) was separated by tlc (system d) into bands corresponding to squalene and cholesterol.

Purification of ^{3}H,^{14}C-Squalene. The squalene zone from above (7.1 μCi of ^{14}C) was eluted with benzene and purified by tlc in solvent system a. After elution of the squalene band (3.3 μCi of ^{14}C) an aliquot was mixed with nonradioactive squalene and purified *via* the thiourea clathrate.[20] On reisolation the squalene had a ^{3}H:^{14}C ratio of 8.48.

Purification of ^{3}H,^{14}C-Cholesterol. The cholesterol band from above (13.9 μCi of ^{14}C) was eluted with acetone and twice purified by tlc. The plates were developed two and three times, respectively, with solvent system e. Extraction of the cholesterol zone with chloroform in a Soxhlet extractor for 6 hr afforded cholesterol (7.8 mg, 9.2 μCi of ^{14}C, ^{3}H:^{14}C ratio 5.02). A sample of the cholesterol (7.21×10^4 dpm of ^{14}C) was mixed with nonradioactive material (75 mg) and purified *via* the dibromide.[21] The purified material was crystallized (CH_3OH) to constant specific activity and ^{3}H:^{14}C ratio (see Table II).

Table II. ^{14}C-Specific Activity and ^{3}H:^{14}C ratio of Cholesterol Biosynthesized from 4*R*-(2-^{14}C,4-^{3}H)-MVA (^{3}H:^{14}C Ratio 8.03) by Rat Liver Enzymes

Crystalln[a]	^{14}C specific activity, dpm/mg	^{3}H:^{14}C ratio—Isotopic	^{3}H:^{14}C ratio—Atomic
		5.02	3.12:5
1	74.7	4.72	2.94:5
2	71.2	4.94	3.08:5
3	70.7	4.92	3.06:5
4	71.0	4.93	3.07:5

[a] Crystallized from methanol.

Preparation of an Acetone Powder of Bovine Adrenal Mitochondria. Twenty adrenal glands were dissected and the cortices (100 g) were homogenized in supplemented sucrose[9] (2.5 ml/g of tissue). The homogenate was centrifuged at 12,500*g* for 25 min and the combined pellets, containing cell debris, nuclei, and mitochondria,[22] were suspended in 0.1 *M* KCl (160 ml). The suspension was poured into acetone (10 vol) at −20° and the resultant powder (14.8 g) was collected by filtration, washed with ether, dried, and stored[23] at −10°.

Cleavage of ^{3}H,^{14}C-Cholesterol to Isocaproic Acid. The acetone powder of bovine adrenal mitochondria (5.0 g) was stirred with 0.02 *M* phosphate buffer (100 ml, pH 7.4) for 1.5 hr. The mixture was suspended thoroughly with a Potter-Elvehjem homogenizer and the volume made up to 233 ml with further phosphate buffer. To the suspension was added a solution of 3β-hydroxychol-5-enoic acid (11.22 mg) in dimethylformamide (1.0 ml).[23]

To three 500-ml Erlenmeyer flasks were added the above enzyme preparation (78 ml), biosynthetic ^{3}H,^{14}C-cholesterol (2.66 μCi of ^{14}C, 2.27 mg) suspended in water (2.0 ml) with Triton X-100 (Rohm & Haas, Co., Philadelphia, Pa., 50 mg), and an NADPH generating system comprising NADP (25 mg), glucose-6-phosphate dehydrogenase (100 enzyme units) dissolved in 0.02 *M* phosphate buffer, and the mixture was incubated at 37° for 2 hr.

Isolation of 3*R*-(1,5-$^{14}C_2$-3-$^{3}H_1$)-Isocaproic Acid. The incubation mixtures in the three flasks were combined and acidified with dilute phosphoric acid to congo red. The mixture was steam distilled and 1.5 l. of distillate was collected. The distillate was saturated with NaCl, cold isocaproic acid (254 mg) was added, and the solution was extracted continuously with ether (48 hr). A total of 5.05×10^5 dpm of ^{14}C was recovered from the ether extract. Addition of another 200 mg of cold isocaproic acid to the aqueous solution and further continuous extraction (24 hr) gave only negligible amounts of radioactivity. The ether solution was dried, reduced to about 180 ml, and treated with a small excess of an ethereal diazomethane. The excess of diazomethane was destroyed with an ether solution of nonradioactive isocaproic acid and the solution of the ester **9b** was used for degradation.

Preparation of 3*R*-(1,5-$^{14}C_2$,3-$^{3}H_1$)-4-Methyl-1,1-diphenylpent-1-ene (10). (a) The ethereal solution of the ester described above was added to a stirred solution of phenylmagnesium bromide (75 mmol) in ether (100 ml). A white solid separated immediately. Addition of a small sample of the supernatant to hydrochloric acid did not cause evolution of gas. Consequently, an additional 150 mmol of reagent was added. The reaction mixture was refluxed for 2.0 hr and worked up as described previously.

(b) Dehydration of the crude product was carried out in the manner described earlier. The oil thus obtained was distilled through a 6 in. Vigreux column and after an initial forerun, the fraction boiling at 120–130° (0.1 mm) (1.82 g) was collected. The highly colored residue was discarded.

Analysis of the fraction, bp 120–130° (0.1 mm), by glc (column i, 225°) indicated that this was essentially a mixture of the diphenylalkene (**10**) and biphenyl. The diphenylalkene (**10**) was isolated by preparative glc (1.1 g). This material was ozonized without further purification (see below).

An aliquot of the diphenylalkene was diluted with a nonradioactive specimen and was purified further by preparative tlc (benzene–hexane, 1:3). It showed $^{3}H/^{14}C$ = 4.31, ^{14}C specific activity of 249 dpm ^{14}C/mg.

2*S*-(4-^{14}C,2-$^{3}H_1$)-Isovaleraldehyde. A solution of the radioactive diphenylalkene (900 mg) (**11**) was ozonized and the ozonide was converted to isovaleraldehyde as described previously for the nonradioactive specimen. The distillation procedure, however, was slightly modified in that cold isovaleraldehyde was added prior to each distillation (a total of 200 mg). The resulting distillate (100 ml) containing the ^{14}C,^{3}H-isovaleraldehyde was dried and used in the Baeyer–Villiger oxidation.

A portion of the aldehyde was converted to a dimedone derivative. Dimedone (100 mg) was dissolved in methanol (5.0 ml) and the pH of the solution was adjusted to 6.0–6.5 with 2 *N* acetic acid.[18] To the warm solution of the dimedone an aliquot (5 ml) of the above distillate was added and the mixture was refluxed for several minutes. The volatile components were removed *in vacuo*. The residual solid was digested with ether and the ether-soluble portion was fractionated on tlc (benzene, developed twice). The zone corresponding to the dimedone derivative of 2*S*-(4^{14}C,2^{3}H)-isovaleraldehyde was isolated (ethyl acetate). It was crystallized from aqueous ethanol and showed $^{3}H/^{14}C$ = 8.46 and mp 152–153°.

1*S*-(3-^{14}C,1-^{3}H)-Isobutyl Formate (12a) and 1*S*-(3-^{14}C,1-^{3}H)-Isobutyl Alcohol (12b). The solution of ^{14}C,^{3}H-isovaleraldehyde prepared above was converted to (3-^{14}C,1-^{3}H)-isobutyl alcohol by the procedure described earlier for the nonradioactive specimen. The product was subjected to short-path distillation furnishing 1*S*-(3-^{14}C,1-^{3}H)-isobutyl alcohol (120 mg), bp 115–120° (1 atm) (bath temperature). The specimen was homogeneous by glc on columns i and ii and showed $^{3}H/^{14}C$ = 7.90 (specific activity, 64 dpm of ^{14}C/mg).

Enzymatic Oxidation of 1*S*-(3-^{14}C,1-^{3}H)-Isobutyl Alcohol to (3-^{14}C,1-^{3}H-Isobutyraldehyde (13). A solution of NAD^+ (360 mg) and yeast alcohol dehydrogenase (12 mg) in 30 ml of glycine–sodium hydroxide buffer (pH 9.9 (25°)) was prepared. An aliquot (0.1 ml) of the above solution was diluted tenfold with the buffer and was used as the blank for the optical density measurement.

To the remaining solution was added (3-^{14}C,1-^{3}H)-isobutyl alcohol (20 mg, 312 μmol). Aliquots (0.5 ml) were removed at intervals and diluted tenfold with the buffer, and their optical densities were recorded. Usually after 4 hr the optical density became constant (2.2) and this corresponded to the formation of 110 μmol of NADH. The pH of the solution was adjusted to about 6.5 with

(20) *Cf.* J. M. Zander, J. B. Greig, and E. Caspi, *J. Biol. Chem.*, **245**, 1247 (1970); L. J. Goad and T. W. Goodwin, *Biochem. J.*, **99**, 735 (1966).

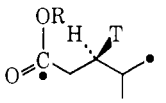
(21) J. M. Zander and E. Caspi, *J. Biol. Chem.*, **245**, 1682 (1970); L. Fieser, "Organic Syntheses," Collect. Vol. IV, Wiley, New York, N. Y., 1963, p 196.

(22) T. Kimura, P. S. Satoh, and T. T. Tchen, *Anal. Biochem.*, **16**, 355 (1966).

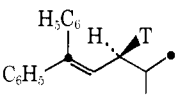
(23) P. R. Raggatt and M. W. Whitehouse, *Biochem. J.*, **101**, 819 (1966).

dilute acetic acid, and a solution of dimedone (100 mg) in hot water (5.0 ml) was added. The mixture was heated at 70–80° for 15 min, left at room temperature (4–5 hr), and stored at 0° for 16 hr. The obtained solid was collected by filtration and washed with water. The filtrate and the washings were discarded. The solid material was digested with several portions of hot ethanol and the ethanol-soluble residue was purified by tlc (benzene, developed twice). The zone corresponding to the dimedone derivative of isobutyraldehyde was isolated (ethyl acetate) (8.5 mg) and was crystallized twice from aqueous ethanol furnishing the dimedone derivative of (3-^{14}C,1-^{3}H)isobutyraldehyde, mp 154°, $^{3}H/^{14}C$ = 8.26 (lit.[16] 154°). In an analogous experiment carried out first, the dimedone derivative showed a $^{3}H:^{14}C$ ratio of 6.31.

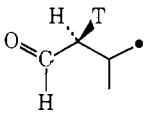
(3-^{14}C)-Methyl isobutyrate (14)–(2-^{14}C,1-^{3}H)-isobutyl alcohol (40 mg) was added in drops to the stirred yellow solution of ru-



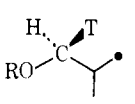
9a, R = H
b, R = CH_3



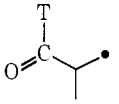
10



11



12a, R = HCO
b, R = H



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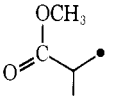

14

thenium tetroxide prepared by stirring ruthenium dioxide (10 mg) in water (5.0 ml) with a solution of sodium metaperiodate (80 mg) in water (5.0 ml).[24] A solution of sodium metaperiodate (250 mg) in water (3.0 ml) was used for regeneration of the ruthenium tetroxide.[4a] After the addition of the alcohol was complete, a sufficient amount of the sodium metaperiodate solution was added to give a yellow solution. After 30 min (when the yellow color still persisted) the excess reagent was decomposed with isopropyl alcohol. The insoluble solid was removed by filtration and washed with small amounts of water. The filtrate was made alkaline with a 1.5 N sodium hydroxide solution, and was washed with ether (three 25-ml portions). The aqueous phase was then acidified with concentrated hydrochloric acid, saturated with salt, and extracted with ether (five 25-ml portions). The combined ether extract was washed twice with a saturated sodium chloride solution, dried, and concentrated by distillation through a 2-ft column packed with glass helices. The concentrate (*ca.* 3.0 ml) was esterified with diazomethane and fractionated by preparative glc (70°, column i). The eluate corresponding in retention time to the authentic material was trapped in toluene (8 ml) cooled in Dry Ice–acetone. The toluene solution containing (3-^{14}C)-methyl isobutyrate was distilled to dryness at atmospheric pressure and the distillate was made up to 10 ml with toluene.

A threefold concentrated scintillating solution was prepared by diluting 12.6 ml of "Liquifluor" (25-fold concentrated) with 100 ml of toluene. The sample for counting was prepared by adding the whole of the ester solution (10 ml) to the phosphor (5 ml). It contained 1860 dpm of ^{14}C and no tritium ($^{3}H/^{14}C$ = 0.019).

Results and Discussion

In the initial planning stage of this work an assumption was made that, by analogy with many other enzymatic reactions, the reduction of Δ^{24} of lanosterol by rat liver enzymes would be a stereospecific process. An indication that in fact this might be the case was provided by the observation that the hydroxylation of a terminal methyl of cholesterol biosynthesized by rat liver enzymes from 2-^{14}C-MVA occurs exclusively on the methyl originating from C-3′ of MVA.[25,26] The four possible pathways of the stereospecific reduction of Δ^{24} of lanosterol were discussed in the introductory portion of the paper, and it was also pointed out that the mechanism of the reduction could be defined by determining the prochirality at C-24 and -25 in cholesterol biosynthesized from 4-*R*-(2-^{14}C,4-^{3}H)-MVA. Our approach was first to determine the stereochemistry at C-24. With this in mind we have incubated the biosynthetic cholesterol with an adrenal enzyme preparation and after considerable efforts succeeded in accumulating sufficient amounts of 1,5-$^{14}C_2$,3-$^{3}H_1$-isocaproic acid.

At this point two problems had to be solved. It was necessary to demonstrate (a) that the degradation of isocaproic acid to isobutyl alcohol could be carried out without disturbing the asymmetry of the isotopic hydrogen atom and (b) the applicability of the NAD$^+$–YADH oxidation for the configurational assignment of 1-^{3}H-isobutyl alcohol. The degradation problem was solved with the use of 3*R*- and 3*S*-3-$^{2}H_1$-isocaproic acids and it was shown that these can be converted to 1*S*- and 1*R*-1-$^{2}H_1$-isobutyl alcohols, respectively, with retention of stereochemistry.[4a,13] We proved also that NAD$^+$–YADH oxidation of isobutyl alcohol proceeds with stereospecific abstraction of the 1-pro-*R* proton. It was noted that the NAD$^+$–YADH oxidation of deuterated isobutyl alcohol, in which a C–^{2}H bond was broken, involved a considerable isotopic effect,[13] and we did not know how to translate this observation to the oxidation of 1-^{3}H-isobutyl alcohols. Consequently we undertook to evaluate this question with the use of 1-^{3}H-isobutyl alcohols.

Isobutyraldehyde was reduced with $LiAl^{3}H_4$ to yield 1*RS*-1-$^{3}H_1$-isobutyl alcohol. Since we were not certain that the isobutyraldehyde did not contain some acidic material which could yield the undesired 1*RS*-1-$^{3}H_2$-isobutyl alcohol we thought it prudent to reoxidize the ^{3}H-isobutyl alcohol to ^{3}H-isobutyraldehyde and then rereduce this with $LiAlH_4$ to the 1*RS*-1-$^{3}H_1$-isobutyl alcohol. The (+)-1*S*-1-^{3}H-isobutyl alcohol was prepared by incubating 1-^{3}H-isobutyraldehyde with fermenting bakers yeast as previously described.[13] Oxidation of the 1*S*-1-$^{3}H_1$-isobutyl alcohol (3.6×10^{10} dpm/mol; as acid phthalate half-ester) gave 1-^{3}H-isobutyraldehyde (3.76×10^{10} dpm/mol; 3.4×10^{10} dpm/mol as dimedone derivative). It is clear therefore that no significant secondary isotope effect is involved in the oxidation of the 1*S*-alcohol.

For the evaluation of a primary isotope effect in the absence of 1*R*-1-^{3}H-isobutyl alcohol we employed 1*RS*-1-^{3}H-isobutyl alcohol (1.98×10^{11} dpm/mol; as acid phthalate) as previously described.[13] Oxidation of the racemic alcohol with NAD–YADH gave 1-^{3}H-isobutyraldehyde (1.07×10^{11} dpm/mol; 1.03×10^{11} dpm/mol; dimedone). Thus in contrast to the oxidation of 1*RS*-1-$^{2}H_1$-isobutyl alcohol which showed a significant isotope effect[13] the oxidation of the tritiated alcohol proceeded without a noticeable isotope effect. That the tritium was located at C-1 of the 1*RS*-1-^{3}H-isobutyl alcohol (3.98×10^{10} dpm/mol; acid phthalate) was proved by oxidizing it to isobutyric acid (1.69×10^{8} dpm/mol) which retained only 0.4% of the isotopic hydrogen. It was now clear that the NAD$^+$–YADH system could be applied to the evaluation of the stereochemistry of the 1-^{3}H-isobutyl alcohol derived from biosynthetic

(24) D. M. Piatak, H. B. Bhat, and E. Caspi, *J. Org. Chem.*, **34**, 112 (1969); D. M. Piatak, G. Herbst, J. Wicha, and E. Caspi, *ibid.*, **34**, 116 (1969).

(25) O. Bersëus, *Acta Chem. Scand.*, **19**, 325 (1965).

(26) K. A. Mitropoulos and N. B. Myant, *Biochem. J.*, **97**, 26c (1965).

cholesterol and that isotope effects would not interfere with the interpretation of results.

The incubation of 4*R*-(2-^{14}C,4-$^{3}H_1$)-MVA (^{3}H:^{14}C ratio 8.03; atomic ratio (ar) 1:1) with a rat liver preparation gave $^{14}C_6$,$^{3}H_6$-squalene (**3**) (^{3}H:^{14}C ratio 8.48; ar 6.34:6) and $^{14}C_5$-17α,20,24*R*-$^{3}H_3$-cholesterol (**2**) (^{3}H:^{14}C ratio 4.93; ar 3.07:5) (Table III). Incubation

Table III. Isotopic and Atomic Ratios (^{3}H:^{14}C) of Cholesterol (and Its Degradation Products) Obtained by Incubation of 4*R*-(2-^{14}C,4-^{3}H$_1$)-MVA with a Rat Liver Homogenate

	^{3}H:^{14}C ratio, dpm	^{3}H:^{14}C atomic ratio Found	Theor
Mevalonic acid	8.03		1:1
Squalene	8.48	6.34:6	6:6
Cholesterol	4.93	3.07:5	3:5
4-Methyl-1,1-diphenyl-1-pentene (**10**)	4.31	1.07:2	1:2
Isovaleraldehyde (**11**)	8.46	1.05:2	1:2
Isobutyl alcohol (**12b**)	7.90	0.92:1	1:1
Methyl isobutyrate (**14**)	0.019	0.002:1	0:1
Isobutyraldehyde (**13**)	6.31	0.79:1	1:1
	8.26	1.03:1	1:1

of the cholesterol with an adrenal enzyme system and isolation of the 1,5-^{14}C,3*R*-3-$^{3}H_1$-isocaproic acid (**9a**) was described in the Experimental Section. Because of scarcity of the ^{14}C,^{3}H-isocaproic acid (**9a**) it was thought advantageous to transform the acid to the methyl ester and without purification convert this *via* a Grignard reaction with phenylmagnesium bromide to the diphenylalkene (**10**). The alkene (**10**) was extensively purified and showed the expected ^{3}H:^{14}C ratio 4.31 (ar 1.07:2). Ozonization of **10** was followed by a reductive work-up to yield 2*S*-$^{3}H_1$,4-$^{14}C_1$-isovaleraldehyde (**11**) which was purified and characterized as the dimedone derivative which had a ^{3}H:^{14}C ratio 8.46 (ar 1.05:1). The aldehyde on Baeyer–Villiger oxidation gave a mixture of 1*S*-1-^{3}H,3-^{14}C-isobutyl alcohol (**12b**) and of the corresponding formate (**12a**). The latter was converted to **12b** by treatment with LAH. The combined isobutyl alcohol **12b** after purification on glc showed a ^{3}H:^{14}C ratio 7.90 (acid phthalate) corresponding to the expected atomic ratio (ar) 0.92:1. The oxidation of the alcohol **12b** with ruthenium tetroxide in water gave 3-^{14}C-isobutyric acid which was counted as the methyl ester (**14**). The ester contained ^{14}C only and was devoid of tritium (^{3}H:^{14}C ratio 0.019; ar 0.002:1) indicating that all the tritium is located at C-1 of **12**. This constitutes the first proof that a proton derived from the 4-pro-*R* position of MVA is indeed located at C-24 of cholesterol as anticipated.

We now exposed the $^{3}H_1$,$^{14}C_1$-isobutyl alcohol **12b** to NAD$^+$ and yeast alcohol dehydrogenase and isolated the isobutyraldehyde. Two oxidation experiments were carried out and the aldehyde[13] was characterized as the dimedone derivative which showed ^{3}H:^{14}C ratios 6.31 and 8.26 (ar 0.79:1; 1.03:1), respectively. The reason for the somewhat low isotope ratio in the first experiment is not clear, but is probably the result of some nonspecific aerial oxidation of a small amount of the alcohol. In any event it is abundantly clear that the enzymatic oxidation of **12** to **13** proceeded with retention of all the tritium at C-1. Since we proved[13] that NAD$^+$–YADH oxidizes isobutyl alcohol to isobutyraldehyde by abstracting the 1-pro-*R* proton it follows that the 1-^{3}H,3-^{14}C-isobutyl alcohol derived from the biosynthetic cholesterol must have the 1*S* stereochemistry. Because of the change of the order of priorities[27] at C-1 of **12** as compared to that at C-24 of cholesterol it follows that cholesterol biosynthesized from 4*R*-(2-^{14}C,4-$^{3}H_1$)-MVA in a rat liver enzyme system must have the 24*R* stereochemistry. Consequently in the reduction of Δ^{24} of lanosterol by rat liver enzymes a 24-pro-*S* proton is added. Hence in the overall reduction of this double bond by a rat liver system the possibilities of front side attack as shown in **7** and **8** need not be considered. Recently[28] it was shown that the newly added proton at C-24 originates from the water of the medium and the equivalent of a "hydride ion" derived from NADPH adds at C-25.

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