Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1037–1045. Pergamon Press. Printed in Great Britain

CHOLESTEROL BIOSYNTHESIS BY RAT LIVER MICROSOMES: CONCERNING C-5 DOUBLE BOND INTRODUCTION

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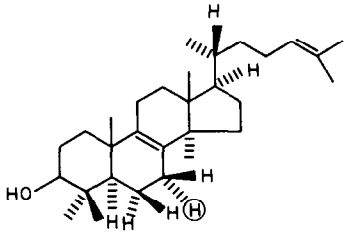
(*Received* 11 *October* 1976)

SUMMARY

The ionic mechanism of dehydrogenation resulting in double bond formation is thought to proceed via the abstraction of a hydride ion and a proton from neighboring carbon atoms. The applicability of such a mechanism to the conversion of 5α-cholest-7-en-3β-ol to cholesta-5,7-dien-3β-ol by a microsomal rat liver acetone powder preparation [16] was investigated. The synthesis of the required substrate [5α-^{3}H]-cholest-7-en-3β-ol is described. Incubation of [5α-^{3}H; $^{14}C_5$]-cholest-7-en-3β-ol with the enzyme preparation in the presence of added AY 9944; NAD^+ and air gave [$^{14}C_5$]-cholesta-5,7-dien-3β-ol. The tritium abstracted from the substrate was found in the water and the recovered NADH contained little tritium. A similar incubation was carried out with the previously prepared [6α-^{3}H; $^{14}C_5$]-5α-cholest-7-en-3β-ol [15]. Again in this instance the abstracted tritium atom was found in the water while the recovered NADH contained an insignificant amount of tritium. The results indicate that both the 5α- and 6α-hydrogen (tritium) atoms removed from the 7-ene in the conversion to the 5,7-diene under the conditions employed are ultimately transferred to the water of the medium. The route by which these hydrogen atoms are abstracted is not clear and requires further study.

INTRODUCTION

The enzymatic transformations of lanosterol (**1**) to cholesterol (**2**) entails the removal of three methyl groups from C-4 and C-14, reduction of the C-24 double bond and the transposition of the C-8(9) double bond to the C-5 position [1]. Although the overall process is fairly well understood the timing and the exact sequence of the reactions is not yet known. In rat livers the introduction of the C-5 double bond seems to occur in the later stages of cholesterol elaboration [2] and apparently requires the presence of the 7(8)-double bond [3]. It is now considered likely that the transformation of (**1**) to (**2**)



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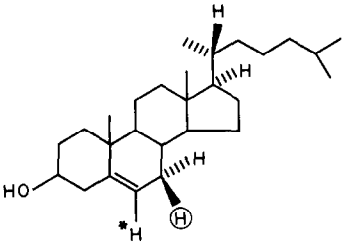
involves, among others, the isomerization of the 8(9) double bond of (**3**) to the 7(8) position in (**4**) [4]. In rat livers this isomerization proceeds via the abstraction of a 7β-hydrogen of (**1**) derived from 2-pro-S of mevalonic acid (MVA) [5–8], and reprotonation at the 9α position [9]. Following the removal of the C-4 gem-dimethyl group the resulting (**5**) is dehydrogenated to yield the 5,7 diene (**6**) [2]. The mechanism of the dehydrogenation is still not well understood. However, it has been established that the process requires oxygen [3, 10] and pyridine nucleotides [10]. It was also proven that the dehydrogenation proceeds



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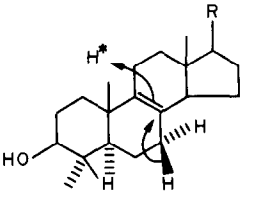

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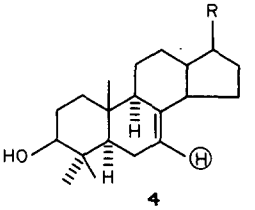

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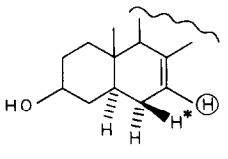

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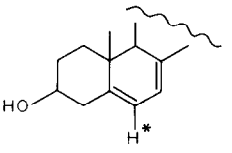

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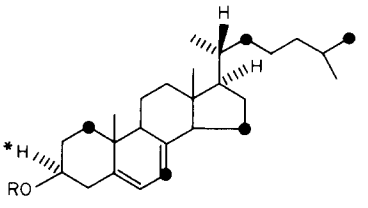

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(a) ● ^{14}C; R=H; $H^*=^1H$
(b) ● ^{14}C; R=Ac; $H^*=^1H$
(c) ● ^{12}C; R=H; $H^*=^3H$
(d) ● ^{12}C; R=Ac; $H^*=^3H$

with the loss of 5α and 6α hydrogen atoms [11]. In this paper we concern ourselves with the fate of the 5α and 6α hydrogen atoms abstracted in the dehydrogenation of cholest-7-en-3β-ol to cholesta-5,7-dien-3β-ol.

EXPERIMENTAL PROCEDURE

Materials. NAD^+, NADH, $NADP^+$ and ATP were purchased from Sigma Chemical Co. The inhibitor [12] AY-9944 was a gift from Dr. D. Dvornick, Ayerst Laboratories, Montreal. Tritiated water, [^{3}H] $NABH_4$[168 mCi/mM] and (3RS,2-^{14}C) mevalonic acid dibenzylethylenediamine salt were purchased from New England Nuclear. Thin layer chromatography (t.l.c.) was carried out on plates coated with silica gel [Merck (HF 254 + 366)]. Dry column chromatography was carried out as previously described [13]. Cholesta-5,7-dien-3β-ol acetate was purchased from Steraloid, Inc., Pawling, NY. The acetate was removed ($LiAlH_4$–ether) and the alcohol purified (t.l.c.) and crystallized before use.

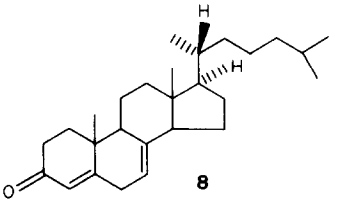
Gas liquid chromatography (g.l.c.) was carried out on a Hewlett–Packard instrument model 7620A equipped with a glass column packed with SE-30 (1%) on a gas chrom-Q (80–100 mesh) support. The injection port temperature was 250°. The column was maintained at 230° and was eluted with He (30 ml per min.).

Physical measurements. Infrared spectra were recorded on compounds incorporated in KBr wafers using Perkin Elmer 237 spectrophotometer. U.V. spectra were recorded in methanolic solutions on a Perkin Elmer 202 instrument. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ solutions on a Varian EM 360 spectrometer at 60 MHz. Peaks are reported in δ units downfield from the tetramethylsilane internal standard. Mass spectra were recorded on a DuPont 21-491 instrument. Melting points were taken on a hot stage and are corrected.

Radioactive samples were counted in a Nuclear Chicago Co. Model Mark II instrument for a sufficient time to assure a counting error no greater than 3%. Steroids were counted in a scintillation solution (10 ml) prepared by diluting Liquifluor (purchased from New England Nuclear Co., Boston, MA) 42 ml to 1000 ml with toluene (spectral grade). Aqueous samples (0.1 ml) were counted in (10 ml) of Aquasol (purchased from New England Nuclear Co., Boston, MA).

The described radioactive compounds were first synthesized without the added isotopes. The non-radioactive products were fully characterized.

[5α-^{3}*H*]-*cholest-7-en-3-one* (**10***b*). (approx. 83% of ^{3}H at 5α; approx. 7% of ^{3}H at 6α; approx. 8% of ^{3}H at 6β-positions, and approx. 2% unaccounted for) (see below). Cholesta-4,7-dien-3-one (**8**) was prepared by Oppenauer oxidation of cholesta-5,7-dien-3β-ol (**7**). The 4,7-dien-3-one (**8**) was exhaustively purified by dry column chromatography [13] followed by t.l.c.



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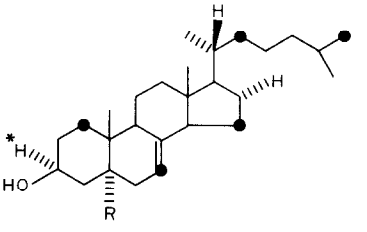
(ethylacetate–hexane (1:9, v/v), and crystallization. To a solution of (**8**) (500 mg) in dry pyridine (4 ml), [^{3}H]-$NaBH_4$ (approx. 25 mCi) was added and the mixture was stirred for 16 h at ambient temperature [14]. To complete the reduction, $NaBH_4$ (150 mg) was added, and the reaction was continued for 5 h. Water (200 ml) was added and the product was recovered with ether (2 × 200 ml). The ether extract was washed with 0.2 N HCl (×2), 5% sodium bicarbonate (×2), water (×3), dried and evaporated. The residue was fractionated by t.l.c. (ethylacetate–hexane (1:9, v/v); developed (×3) and the zone corresponding to (**9**) was re-chromatographed in the same system to yield the [^{3}H]-5α-cholest-7-en-3β-ol (600 μCi, 30 mg). An aliquot (approx. 3.7×10^5



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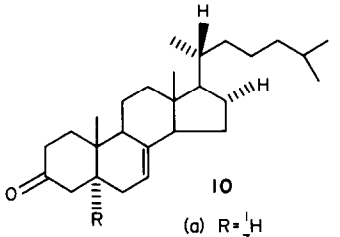
(a) R=^{1}H; ● = ^{14}C; $H^*=^1H$
(b) R=^{1}H; ● = ^{12}C; $H^*=^1H$
(c) R=^{3}H; ● = ^{12}C; $H^*=^1H$
(d) R=^{1}H; ● = ^{12}C; $H^*=^3H$
(e) R=^{3}H; ● = ^{14}C; $H^*=^1H$

d.p.m.) was diluted with cold (**9b**) (33 mg) and crystallized (methanol–chloroform) (× 3). The S.A. of the crystallized material was constant (1.0×10^4 d.p.m. per mg).

The obtained tritiated-5α-cholest-7-en-3β-ol (approx. 600 μCi) was dissolved in acetone (2.5 ml) and treated with Jones reagent (0.1 ml) for 10 min at ambient temperature. Following the conventional workup and t.l.c. fractionation (ethylacetate–hexane (1:9 v/v)), the 5α-cholest-7-en-3-one was obtained (approx. 200 μCi). The [^{3}H]-5α-cholest-7-en-3-one was dissolved in 5% methanolic KOH (30 ml) and the solution was refluxed for 2.5 h under N_2. An aliquot of the recovered equilibrated [5α-^{3}H]-cholest-7-en-3-one (**10b**) (approx. 6×10^5 d.p.m.) was diluted with nonradioactive 5α-cholest-7-en-3-one (**10a**) (90 mg). A portion of the dilute sample (30 mg) was crystallized (× 3) and showed a constant S.A. (6.8×10^3 d.p.m. of ^{3}H per mg).



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(a) R = ^{1}H
(b) R = ^{3}H

The remaining portion of the dilute [5α-^{3}H]-3-ketone (**10b**) (60 mg) was equilibrated with 5% methanolic KOH (30 ml) for 5 h under N_2. The recovered product was crystallized (× 3) and showed a constant S.A. (7×10^3 d.p.m. of ^{3}H per mg).

[5α-^{3}*H*]-*cholest-7-en-3β-ol* (**9c**). (approx. 83% of ^{3}H at 5α; approx. 7% of ^{3}H at 6α-; approx. 8% of ^{3}H at 6β-positions; and approx. 2% unaccounted for) (see below).

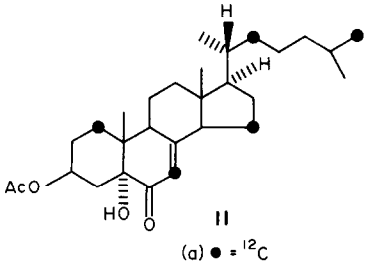
The [5α-^{3}H]-cholest-7-en-3-one (**10b**) was dissolved in dry ether (50 ml) then LAH (excess) was added and the mixture was stirred for 2 h at room temperature. The reaction was terminated with ethanol, then water was added and the solid removed by filtration. The aqueous phase was separated and extracted with ether (× 2). The combined ether solution was washed, dried and concentrated. The obtained residue was fractionated by t.l.c. (ethylacetate–hexane (1:9 v/v)) and the zone corresponding to [5α-^{3}H]-cholest-7-en-3β-ol was recovered and crystallized to yield (**9c**) (approx. 100 μCi; 13 mg). An aliquot of the [5α-^{3}H]-3β-ol (**9c**) (approx. 2.7×10^5 d.p.m.) was diluted with nonradioactive material (**9b**) (27 mg) and, on crystallization, showed a constant S.A. (9.7×10^3 d.p.m. of ^{3}H per mg).

[3α-^{3}*H*]-5α-*cholest-7-en-3β-ol* (**9d**). To a solution of (**10a**) (25 mg) in methanol (10 ml) and ether (3 ml), [^{3}H]-$NaBH_4$ (approx. 1.3 mCi) was added and the mixture was stirred for 1 h at room temperature. Then $NaBH_4$ (50 mg) was added and after 30 min the reaction was terminated by the addition of a 10% solution of NH_4Cl. The product was recovered (ether) and purified by t.l.c. to yield [3α-^{3}H]-5α-cholest-7-en-3β-ol (**9d**) (17 mg, 600 μCi). An aliquot of [3α-^{3}H]-3β-ol-(**9d**) (approx. 2.2×10^5 d.p.m.) was diluted with cold material (**9b**) (22 mg) and, on crystallization showed a constant S.A. (10.5×10^3 d.p.m. per mg).

To a solution of the [3α-^{3}H]-3β-ol-(**9d**) (1.8×10^5 d.p.m. of ^{3}H) in acetone (2.5 ml) nonradioactive 7-en-3β-ol (**9b**) (30 mg) was added and the mixture was treated with Jones reagent (1 drop) for 10 min at room temperature. The product was recovered and purified by t.l.c. The obtained 5α-cholest-7-en-3-one contained 1.6×10^4 d.p.m. This indicates that more than 90% of the tritium was present in the 3α position of the [3α-^{3}H]-5α-cholest-7-en-3β-ol (**9d**).

Determination of tritium distribution in [5α-^{3}*H*]-*cholest-7-en-3β-ol* (**9c**)

[$^{14}C_5$]-*cholest-7-en-6-one-3β,5α-diol 3-acetate* (**11b**). An aliquot of the 5,7-dien-acetate (**7b**) (30 mg) (4.3×10^3 d.p.m. of ^{14}C; ^{3}H:^{14}C ratio 10.0) enzymatically derived from [5α-^{3}H, $^{14}C_5$]-**9e**, was oxidized with Jones reagent (5 min at 0°). The resulting [$^{14}C_5$]-cholest-7-en-6-one-3β,5α-diol 3-acetate (**11b**) was mixed with authentic material (**11a**) and crystallized (× 3). The dilute (**11b**) showed a S.A. of 120 d.p.m. of ^{14}C per mg; and ^{3}H:^{14}C ratio 2.05. This indicates that about 8% of the tritium in the [5α-^{3}H]-(**9c**) was in the 6β position.



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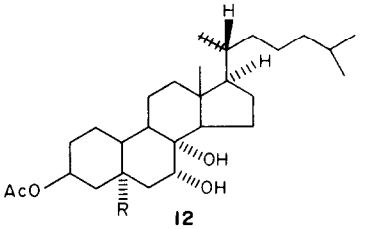
(a) ● = ^{12}C
(b) ● = ^{14}C

[5α-^{3}*H*]-*cholesta-3β,7α,8α-triol* 3-*acetate* (**12b**). A sample of [5α-^{3}H]-cholest-7-en-3β-ol acetate (prepared from the alcohol [5α-^{3}H]-**9c**) was added to a solution of nonradioactive (**9b**)-Ac. (750 mg) in chloroform. The mixture was stirred for 10 min, then the solvent was removed in a stream of nitrogen. An aliquot of the residue (50 mg) was crystallized (chloroform–methanol) several times and counted. It contained 1.2×10^3 d.p.m. of ^{3}H per μmol.

The rest of the material was dissolved in ether (25 ml), pyridine (2.5 ml) and osmium tetroxide (850 mg) were added and the mixture was stored 72 h at ambient temperature in the dark [18]. Then pyridine (16 ml) sodium bisulfite (1g) and water (20 ml) were added and the reaction was stirred for 30 min. The mixture was diluted with water, the product was recovered (chloroform) and processed in the usual manner. The obtained residue was freed of traces of pyridine and fractionated by t.l.c. (ethylacetate–hex-

ane (7:3)) to yield (**12b**) (650 mg). The (**12b**) was crystallized and counted. The obtained (**12b**) contained 1.2×10^3 d.p.m. of ^3H per μmol.

[5α-^3H]-*cholest-7-one-3β-ol acetate* (**13a**). Jones reagent was added to a solution of [5α-^{3}H]-3β,7α,8α-triol 3-acetate-(**12b**) (440 mg) in acetone (30 ml) until the color persisted for 3 min [19]. The reaction was terminated with sodium bisulfite, diluted with water, and the product was recovered (chloroform) and processed. The resulting residue was fractionated by t.l.c. (ethylacetate hexane (1:4) v/v) to yield (**13a**) (135 mg). A sample was crystallized (methanol–chloroform) several times and counted. The product (**13a**) contained 1.2×10^3 d.p.m. of ^3H per μmol.



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(a) R = ^{1}H
(b) R = ^{3}H

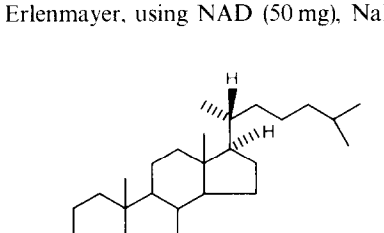
Base equilibration of (**13a**)

A solution of (**13a**) (60 mg) in 5% methanolic KOH (30 ml) was refluxed (3h) under nitrogen in the dark. The recovered sterols were divided in equal portions. One portion was acetylated, and the resulting (**13b**) was crystallized several times and counted. The obtained (**13b**) had a S.A. 1.0×10^3 d.p.m. of ^3H per μmol.

The second portion was reequilibrated as above (5% methanolic KOH (30 ml) reflux (3h) under N_2 in the dark). The recovered product was acetylated and the resulting (**13c**) was crystallized and counted. The S.A. of (**13c**) was 1.0×10^3 d.p.m. of ^3H per μmol.

Preparation of [4*RS*-^3H]-*NADH*

The [4RS-^{3}H]-NADH was prepared in a modified Warburg Erlenmayer, using NAD (50 mg), $NaHCO_3$ (27 mg), tritiated water (1 ml; 1 mCi) and $Na_2S_2O_4$ (50 mg). The mixture was incubated at 37° in an atmosphere of CO_2 until the evolution of the gas ceased (30 min). Following the conventional workup, the tritiated NADH (50 ml) was recovered. An aliquot of the dry powder was dissolved in a 1% solution of $NaHCO_3$ and its absorbancy at 340 nm was determined. Based on ϵ 6250 for NADH, the obtained powder (50 mg) contained about 24% of NADH and 2.2×10^5 d.p.m. of ^3H per mg of the solid.



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(a) R = ^{3}H
(b) R = ^{3}H
(equilibrated x 1)
(c) R = ^{3}H
(equilibrated x 2)

(1,7,15,22,26)[$^{14}C_5$]-5α-*cholest-7-en-3β-ol* (**9a**). The preparation of the $^{14}C_5$-labeled (**9a**), (S.A. 8.7×10^5 d.p.m. of ^{14}C per mg) was previously described [15].

Enzyme preparations and incubations

Incubation of [3α-^3H]-5α-*cholest-7-en-3β-ol* (**9d**) *with a washed rat liver microsomal preparation.* Livers from female Sprague–Dawley rats were homogenized in 0.1 M phosphate buffer containing $MgCl_2$ (4 mM); nicotinamide (30 mM), EDTA (0.1 mM), AY-9944, (0.05 mM). The microsomes were prepared as previously described [15]. For the incubation the microsomal pellet (from 10 g of liver) was suspended in the same buffer solution (10 ml) and the steroid, [3α-^{3}H]-5α-cholest-7-en-3β-ol (**9d**) (approx. 400 μg; approx. 3 μCi of ^3H) was added as an emulsion in the buffer (0.5 ml) and Tween 80 (20–25 mg). The emulsion was prepared by adding to a solution of the steroid in 2 drops of acetone first the Tween 80 and then the buffer (0.5 ml). After the mixture was emulsified by shaking, the acetone was removed in a gentle stream of nitrogen. The incubation was carried out in a Dubnoff shaker for 3 h at 37°C in the air. The reaction was terminated with 10% methanolic KOH (40 ml) and the mixture was refluxed for 3 h under N_2 in the dark. The product was recovered with hexane and was processed in the conventional manner. The crude residue was diluted with non-radioactive 5,7-dien-ol (**9b**) (5 mg) and the mixture was acetylated (0.2 ml pyridine, 0.3 ml acetic anhydride, 16 h at ambient temperature in the dark). The acetates were fractionated by argentation t.l.c. (silica gel–15% $AgNO_3$; ethyl ether–petroleum ether (30–60°) (1:24)). The zones corresponding to the [3α-^{3}H]-cholesta-5,7-dien-3β-ol acetate (**7d**) and [3α-^{3}H]-5α-cholest-7-en-3β-ol acetate (**9d**-Ac) were recovered and rechromatographed (silica gel 15% $AgNO_3$; ethyl acetate–hexane (1:9)). The obtained [3α-^{3}H]-cholesta-5,7-dien-3β-ol acetate was diluted with nonradioactive material and crystallized to constant S.A. Based on the tritium found in the isolated [3α-^{3}H]-cholesta-5,7-dien-3β-ol acetate (**7d**) about 13.3% of the [3α-^{3}H]-7-en-3β-ol (**9d**) was dehydrogenated to (**7d**).

Incubation of [5α-^3H: $^{14}C_5$]-*cholest-7-en-3β-ol* (**9e**) *with a washed rat liver microsomal preparation.* Samples of [$^{14}C_5$]-5α-cholest-7-en-3β-ol (**9a**) (approx. 350 μg; 3.08×10^5 d.p.m. of ^{14}C) and [5α-^{3}H]-cholest-7-en-3β-ol (**9c**) (approx. 1.28 mg approx. 2.8×10^7 d.p.m. of ^3H) were dissolved in ethyl acetate, stirred,

Table 1. Dehydrogenation of [5α-^{3}H; $^{14}C_5$]-cholest-7-en-3β-ol (**9e**) (^{3}H:^{14}C ratio 89.5) to [$^{14}C_5$]-cholesta-5,7-dien-3β-ol (**7a**) by a washed rat liver microsomal preparation

Experiment	[^{3}H] (d.p.m. × 10^6) present in (**9e**) used for incubation	Conversion (%) of (**9e**) to (**7a**) calculated from total ^{14}C in (**7b**)	Calculated amount of [^{3}H] (d.p.m. × 10^5) abstracted	[^{3}H] (d.p.m. × 10^5) found in water	[^{3}H] (d.p.m.) found in NADH
1	5.2	14.1	6.6	6.0	5000
2	5.5	10.1	4.9	3.9	500
3	4.2	13.3	5.0	3.6	1250

and the solution was taken to dryness. Then, a stock acetone solution of the doubly labelled [5α-^{3}H, $^{14}C_5$]-(**9e**) was prepared and aliquots were removed as needed. A sample of the doubly labeled material [5α-^{3}H, $^{14}C_5$]-(**9e**) was diluted with nonradioactive 5α-cholest-7-en-3β-ol (50 mg) and crystallized four times (chloroform–methanol).

The diluted sample of (**9e**) showed a ^{14}C-S.A. of 240 d.p.m. per mg and a H:^{14}C ratio 1 × : 88.96; 2 × : 89.20; 3 × : 89.57; 4 × : 89.45.

An aliquot of the [5α-^{3}H; $^{14}C_5$]-cholest-7-en-3β-ol (**9e**) (4.3 × 10^6 d.p.m. of ^{3}H; ^{3}H:^{14}C ratio 89.5) was emulsified and added to a microsomal preparation as described above. The incubation was carried out in a Dubnoff shaker for 3 h at 37°C in the air. At the termination of the reaction NADH (50 mg) was added and the flask was immediately heated in a boiling water bath for 75 s. The mixture was centrifuged (5 min) at 1000 × *g*. and the supernatant was filtered through celite. The clear filtrate was freeze dried and the distillate was collected in a container cooled in liquid N_2. The collected water (13 ml) contained 6 × 10^5 d.p.m. of ^{3}H.

The residual powder in the distillation flask was processed as previously described [15] and the NADH was isolated. Based on U.V. absorption, 20 mg of NADH was recovered which contained approx. 2000 d.p.m. of ^{3}H which when corrected for 100% recovery corresponds to 5000 d.p.m. of ^{3}H.

The pellet of the 1000 × *g* centrifugation and the celite through which the supernatant was filtered were combined, slurried with buffer (10 ml) then 10% methanolic KOH (40 ml) was added and the mixture was refluxed in the dark under N_2 for 3 h. The sterols were recovered (hexane) in the conventional manner. Following acetylation and t.l.c. fractionation 5α-cholest-7-en-3β-ol acetate and cholest-5,7-dien-3β-ol acetate (**7b**) were isolated, diluted with nonradioactive material and crystallized to constant specific activity. The results are summarized in Table 1.

Determination of the exchange of tritium from [4RS-^{3}H]-NADH during incubation with the microsomal preparation. The experiment was carried out as described above except that nonradioactive 5α-cholest-7-en-3β-ol (**9b**) (250 μg) and [4RS-^{3}H]-NADH (4.8 × 10^5 d.p.m. of ^{3}H) were used. At the termination of the incubation (3 h), NADH (50 mg) was added and the reaction mixture was processed as above. The recovered NADH (25 mg) contained 1.06 × 10^5 d.p.m. of ^{3}H which when corrected for 100% recovery corresponds to 2.1 × 10^5 d.p.m. of ^{3}H. The distilled water contained 2.8 × 10^5 d.p.m. of ^{3}H. Hence 45% of the initial radioactivity was retained in NADH and 55% was exchanged with water.

Procedure for the conversion of 5α-cholest-7-en-3β-ol to cholesta-5,7-dien-3β-ol by a rat liver microsomal acetone powder preparation. The rat liver microsomal acetone powder was prepared by the method of Scallen *et al.* [16]. The acetone powder (30 mg) was suspended in 3 ml of phosphate buffer (pH 7.4) containing magnesium chloride (4 mM) nicotinamide (30 mM) EDTA, (0.1 mM) and AY-9944 (0.05 mM). The NAD (10 mg) was added as a solution in buffer (0.5 ml). The [3α-^{3}H]-(**9d**) was added as an emulsion in Tween 80. The final vol. of the incubation mixture was made up to 5.0 ml with buffer. The incubation was carried out in a Dubnoff shaker for 3 h in the dark at 37°. The reaction mixture was processed as described above except that the saponification time was reduced to 1 h. The cofactor requirements for the reaction are given in Table 2. Control experiments establishing the rate of exchange of tritium from [4RS-^{3}H] NADH with the medium are summarized in Table 3.

*Dehydrogenation of [5α-^{3}H; $^{14}C_5$]-cholest-7-en-3β-ol (**9e**) to [$^{14}C_5$]-cholesta-5,7-dien-3β-ol (**7a**) with a rat liver microsomal acetone powder preparation.* Rat liver microsomal acetone powder (100 mg) was suspended with the use of a glass hand homogenizer in the above

Table 2. Dehydrogenation of [3α-^{3}H]-5α-cholest-7-en-3β-ol (**9d**) to [3α-^{3}H]-cholesta-5,7-dien-3β-ol (**7c**) by a rat liver microsomal acetone powder preparation: evidence for pyridine nucleotide and oxygen requirement

Acetone powder	NAD^+	Gas phase	% Conversion* of (**9d**) to (**7c**) counted as (**7d**)
30 mg	10 mg	Air	11.4
30 mg	nil	Air	<0.1
30 mg	10 mg	Helium	0.7
30 mg	10 mg	Nitrogen	0.25
30 mg heat denatured	10 mg	Air	<0.1

* The incubations were carried out with the same batch of acetone powder for 3 h at 37°. For details see experimental section.

Table 3. The yield of conversion of (**9d**) to (**7c**) and the exchange of tritium from [4-^{3}H] NADH during the incubations with the rat liver microsomal acetone powder preparation

Time (min)	% conversion of (**9d**) to (**7c**) counted as (**7d**)	% of [^{3}H] (from 4-^{3}H NADH) found in the water of the medium
15	1.1	0.0
30	2.2	17.3
60	3.5	28.5
180	4.7	45.0

The experiments were carried in parallel with powder from the same batch, for the indicated periods of time. For the determination of the conversion of the mono-ene to the diene (**7c**) the [3α-^{3}H] (**9d**) was used as substrate and NAD$^+$ was added. The %-conversion was calculated from the amount of [^{3}H] in (**7d**). For the determination of the exchange of tritium, non-radioactive (**9b**) was incubated in the presence of NAD$^+$ and [4RS-^{3}H] NADH.

described mixed phosphate buffer (15 ml). The enzyme suspension was distributed equally in five (25 ml) Erlenmeyer flasks. To each flask, a solution of NAD (10 mg) in the mixed buffer (0.5 ml), was added. The substrate [5α-^{3}H, $^{14}C_5$]-(**9e**) (7.59×10^4 d.p.m. of ^{14}C; ^{3}H:^{14}C ratio 95.3) was emulsified (Tween 80) in the buffer (2.5 ml) then distributed equally among the flasks. The flasks were loosely covered with aluminium foil and incubated in a Dubnoff shaker at 37° for 30 min in the air. At the end of the incubation, the contents of the flasks were combined, 50 mg of NADH was added and the mixture heated in a boiling water bath for 75 s. The reaction was processed as described above and the results are summarized in Table 4.

Dehydrogenation of [6α-^{3}*H*; $^{14}C_5$]-5α-*cholest-7-en-3β-ol with a rat liver microsomal acetone powder preparation.* The [6α-^{3}H]-5α-cholest-7-en-3β-ol [15] was mixed with [$^{14}C_5$]-5α-cholest-7-en-3β-ol (**9a**) [15]. An aliquot of the mixed sample was diluted with cold 5α-cholest-7-en-3β-ol (30 mg) and crystallized three times. It showed a constant S.A. (253 d.p.m. per mg of ^{14}C) and a constant ^{3}H:^{14}C ratio (61.5). The [6α-^{3}H; $^{14}C_5$]-5α-cholest-7-en-3β-ol (6.8×10^6 d.p.m. of ^{3}H ^{3}H:^{14}C ratio 61.5; approx. 403 μg) was incubated with the rat liver microsomal acetone powder preparation as described for (**9e**). The recovery of sterols, NADH and water was carried out as described above. The results are summarized in Table 5.

RESULTS AND DISCUSSION

From a mechanistic point of view, an ionic dehydrogenation reaction resulting in a C–C double bond formation involves the abstraction of a hydride ion and a proton from neighboring carbon atoms. In biological systems, the nucleotides NAD and NADP may function as the hydride ion acceptors. It was previously demonstrated that the conversion of the 7-ene (**5**) to the 5,7-diene (**6**) by a rat liver microsomal preparation requires *inter alia* the presence of NAD or NADP [10]. Hence, should the introduction of the C-5 (6) double bond follow the conventional ionic route, one of the hydrogens could be abstracted with its bonding electrons and transferred to the pyridine nucleotide. The other hydrogen could then be eliminated as a proton, and should end up in the water of the medium. The objective of the present study was limited to the testing of this hypothesis.

Several years ago, we have shown with the use of a washed rat liver microsomal preparation that the abstracted 6α-hydrogen (tritium) atom was not transferred to NAD. We have located the removed tritium atom in the water of the medium [15]. We now report

Table 4. Dehydrogenation of [5α-^{3}H; $^{14}C_5$]-cholest-7-en-3β-ol (**9e**) to [$^{14}C_5$]-cholesta-5,7-dien-3β-ol (**7a**) with a rat liver microsomal acetone powder preparation: S.A. (d.p.m. per mg of ^{14}C) and ^{3}H:^{14}C ratio

Compound and amount of ^{14}C (d.p.m. × 10^4)	Crystallization 1		2		3		4		Average ^{3}H:^{14}C Ratio
	S.A.	Ratio	S.A.	Ratio	S.A.	Ratio	S.A.	Ratio	
(**9e**); 7.59 Starting material	445	94.91	463	95.17	457	95.71	—	—	95.3
(**9e**)-acetate; 6.50 recovered from incubation	342	92.87	372	94.97	372	94.8	398	95.46	94.5
(**7b**); 0.17	87	11.06	82	10.70	81	10.30	83	10.88	10.7
Total [^{3}H] in the water	1.67×10^5 d.p.m.								
Total [^{3}H] in recovered NADH (corrected for 100% recovery)	9.2×10^3 d.p.m.								

The incubation was carried out as described in the experimental section. The sterols were counted at different dilutions.

Table 5. Dehydrogenation of [6α-^{3}H; $^{14}C_5$]-cholest-7-en-3β-ol to [$^{14}C_5$]-cholesta-5,7-dien-3β-ol with a rat liver microsomal acetone powder preparation: S.A. (d.p.m. per mg of ^{14}C) and ^{3}H:^{14}C ratio

Compound and amount of ^{14}C (d.p.m. × 10^4)	Crystallization 1		2		3		4		Average ^{3}H:^{14}C Ratio
	S.A.	Ratio	S.A.	Ratio	S.A.	Ratio	S.A.	Ratio	
[6α-^{3}H:^{14}C]-cholest-7-en-3β-ol 11.6 Starting material	286	62.33	253	61.02	258	61.97	—	—	61.77
Starting material (Ac) 10.0; recovered from incubation	52	63.62	50	60.36	50	60.26	53	61.60	61.46
[$^{14}C_5$]-cholest-5,7-diene-3β-ol acetate 0.20	55	2.22	58	2.18	57	2.20	59	2.11	2.18
Total [^{3}H] in water	1.3 × 10^5 d.p.m.								
Total [^{3}H] in recovered NADH (corrected for 100% recovery)	500 d.p.m.								

The incubation conditions are given in the experimental section. The sterols were counted at different dilutions.

our observations on the fate of the 5α-hydrogen atom. The [5α-^{3}H]-cholest-7-en-3β-ol (**9c**) required for the investigations was synthesized as described in the experimental section, and the obtained [5α-^{3}H] (**9c**) was shown to be identical to authentic samples prepared by two different methods [15, 17]. The [5α-^{3}H]-7-en-3-ol (**9c**) did not contain exchangeable tritium at C-2 and 4. However, the method of preparation of [5α-^{3}H]-(**9c**) via the reduction of (**8**) with [^{3}H] $NaBH_4$-Pyridine did not preclude the possibility of the introduction of some tritium at C-6. Consequently, it was necessary to determine the amounts of tritium present at each of the critically important 5α and 6α positions. We will address ourselves to this point later on (see below).

In preliminary experiments we used [3α-^{3}H]-cholest-7-en-3β-ol (**9d**) which was obtained by reduction of the ketone (**10a**) with [^{3}H]-$NaBH_4$. It was determined that at least 90% of the tritium of (**9d**) was located at the 3α-position.

Incubation of [3α-^{3}H]-(**9d**) (3 μCi) with washed rat liver microsomes in the presence of added NAD (10 mg) and of the AY-9944 inhibitor [12] resulted in the [3α-^{3}H]-cholesta-5,7-dien-3β-ol (**7c**) which was isolated and purified as the acetate. Several incubation experiments were carried out and based on the amount of tritium in the obtained [3α-^{3}H]-5,7-dien acetate (**7d**), the conversion of (**9d**) to (**7c**) proceeded in approx. 11–14% yield.

For the studies of the fate of the 5α-hydrogen, the doubly labeled [5α-^{3}H; $^{14}C_5$]-(**9e**) [^{3}H:^{14}C ratio 89.5] was incubated in a similar manner, with washed microsomes to yield the 5,7-dien-3β-ol (**7a**) which was purified and counted as the acetate (**7b**). The obtained (**7b**) showed a constant S.A. of ^{14}C and had a ^{3}H:^{14}C ratio 9.4 indicating the retention of about 10% of the tritium present in (**9e**). Based on the ^{14}C present in (**7b**) (6.5 × 10^3 d.p.m. of ^{14}C), 13.3% of (**9e**) was converted to the diene. The recovered starting material showed a ^{3}H:^{14}C ratio of 98.0 and this confirmed the reported observations that the C-5 (6) dehydrogenation is accompanied by an isotope effect [11, 15, 18].

To locate the tritium retained in the biosynthesized [^{14}C]-5,7-diene (**7b**), the latter was oxidized with Jones reagent to the [^{14}C]-5α-hydroxy-6-keto (**11b**) which showed a ^{3}H: ^{14}C ratio of 2.05. This establishes that [5α-^{3}H]-(**9c**) and [5α-^{3}H, $^{14}C_5$]-(**9e**) had approx. 90% of tritium distributed between the 5α and 6α positions, 8% in the 6β position, and 2% is unaccounted for.

We chose to determine the amount of tritium at the 6α-position by difference between the total amount of isotopic hydrogen at C-6 and that found at the 6β-position. For the determination of the amount of ^{3}H at C-6, an aliquot of [5α-^{3}H]-(**9c**) was acetylated and the acetate (S.A. 1.2 × 10^3 d.p.m. of ^{3}H per μmol) was treated with osmium tetroxide [18]. The resulting 7α,8α-diol 3β-acetate (**12b**) (S.A. 1.2 × 10^3 d.p.m. of ^{3}H per μmol) was oxidized with Jones reagent to yield the 7-keto-3-acetate (**13a**) (S.A. 1.2 × 10^3 d.p.m. of ^{3}H per μmol) [19]. The sequence of transformations of (**9c**)-acetate via (**12b**) to (**13a**) obviously proceeded without loss of tritium. This establishes the absence of tritium at C-7 of the [5α-^{3}H]-(**9c**) and (**9e**). Base catalyzed equilibration of 7-keto-3-acetate (**13a**), and reacetylation of the product gave (**13b**) (S.A. 1.02 × 10^3 d.p.m. of ^{3}H per μmol). The equilibration to (**13b**) was accompanied by the loss of approx. 15–16% of tritium, which must have been located at C-6. Further equilibration of (**13b**) and acetylation to (**13c**) (S.A. 1.02 × 10^3 d.p.m. of ^{3}H per μmol) proceeded without loss of additional amounts of tritium.

The sequence of the described transformations establishes the distribution of isotopic hydrogen in [5α-^{3}H]-(**9c**) and [5α-^{3}H, $^{14}C_5$]-(**9e**) as follows: approx. 83% of tritium is located at the 5α-position; approx. 8% at the 6β-position; approx. 7% at the 6α-position and approx. 2% is unaccounted for.

Three incubations of the [5α-^{3}H, $^{14}C_5$]-(**9e**) with a fresh rat liver microsomal preparation were carried out. The water of the medium, the NADH and the produced [$^{14}C_5$]-5,7-diene (**7a**) were recovered and counted. The results are summarized in Table 1. The dehydrogenation with fresh rat liver microsomal preparations proceeded in about 10–14% yield (Table 1). It is evident that only a small amount of tritium was present in the NADH while the recovered tritium was located in the water. The discrepancy between the calculated amount of tritium and that detected in the water is probably due to technical experimental errors. It is likely that the isotope effects at the 5α and 6α positions are approximately the same. Should this be the case, it can be calculated that approx. 8% and approx. 92% of the tritium found in the water was derived from the 6α and 5α-tritium atoms respectively. Therefore, for the present discussion, the 8% contribution of tritium from the 6α-position will be disregarded and, as a first approximation, the tritium in the water will be viewed as originating from the 5α-position.

Since our previous [15] and our present results indicate that the 5α and 6α hydrogen (tritium) atoms abstracted from (**9**) were found in the water of the medium, we became concerned whether our washed microsomal preparation indeed required exogenous NAD for the reaction to proceed. To evaluate this point, two incubations of equal amounts of (**9d**), with equal aliquots of a washed microsomal preparation (from the same batch), with and without added NAD (10 mg), were carried out. The reaction proceeded in both instances and while in the presence of added NAD, 11% of [3α-^{3}H]-(**9d**) was desaturated, in the absence of added NAD the yield was 9%. It became clear that in *this* enzyme preparation, the dehydrogenation was not dependent on the added NAD.

We also noticed that the rate of exchange of the C-4 hydrogens of the NADH was considerably higher than expected [15] (see experimental). Thus incubation of nonradioactive (**9b**) in the presence of NAD and [4RS-^{3}H]-NADH resulted in a 55% exchange of tritium with the water of the medium. Only 45% of the tritium remained associated with the recovered NADH. Apparently in this preparation, the enzyme(s) responsible for the exchange of the C-4 hydrogens of the nucleotide was not completely removed. Under the circumstances, it was considered impractical to continue the study with the washed microsomes. Consequently in further investigations, we used a rat liver microsomal acetone powder which was prepared as described by Scallen *et al.* [16].

We first tested the 5,(6)-desaturating capacity of the powder and in particular the dependence of the process on added NAD. The results are summarized in Table 2. Clearly, the dehydrogenation proceeded only in the presence of added NAD and in an atmosphere of air. It was now necessary to evaluate the rate of exchange of the C-4 hydrogen atoms of NADH in this preparation. As can be seen from Table 3, in the first 15 min of incubation approx. 1% of the [3α-^{3}H]-(**9d**) was converted to [3α-^{3}H]-(**7c**) (counted as 3-acetate (**7d**)) and within that period of time the exchange of tritium from the added [4-^{3}H]-NADH was negligible. However, after 30 min approx. 20% of the tritium was already present in the water and this increased to approx. 30% after 60 min. Subsequently the rate of exchange seemed to slow down and after 3 h, only approx. 45% of tritium was exchanged. The observations indicated that the incubation time should not exceed 30 min. In practice these conditions limited *a priori* the expected yield of the diene to approx. 2%.

We now incubated the [5α-^{3}H; $^{14}C_5$]-(**9e**) (^{3}H:^{14}C ratio 95.3) with the resuspended rat liver microsomal powder for 30 min and the diene [$^{14}C_5$]-(**7a**) was obtained in about 2.2% yield (Table 4). The ^{3}H:^{14}C ratio (10.7) of the [$^{14}C_5$]-diene acetate (**7b**) was in accord with previous observations. Again, the tritium abstracted from the [5α-^{3}H, $^{14}C_5$]-(**9e**) (1.5×10^5 d.p.m.) was found in the water (1.67×10^5 d.p.m.). The recovered NADH contained 9.2×10^3 d.p.m. of tritium.

It may be recalled that our previous dehydrogenation studies of the 6α-^{3}H analog of (**9**) were carried out with a washed microsomal preparation [15] and the abstracted 6α-tritium atom was also located in the water. Therefore, we considered it necessary to reexamine the previous results with the presently employed microsomal acetone powder.

The dehydrogenation of the [6α-^{3}H; $^{14}C_5$]-analog of (**9**) (^{3}H:^{14}C ratio 61.77) with the resuspended rat liver microsomal acetone powder proceeded in approx. 2% yield (Table 5). Again, the tritium abstracted from the 6α-position (1.2×10^5 d.p.m.), was found in the water (1.3×10^5 d.p.m.). The amount of tritium associated with the recovered NADH was small, 0.5×10^3 d.p.m. It is apparent that the results with the rat liver microsomal powder are analogous to those obtained previously with the washed microsomal preparation [15].

The reported observations indicate that both the 5α- and 6α-hydrogen atoms removed in the dehydrogenation of (**5**) and (**6**) are ultimately transferred to the water of the medium. It can, therefore, be concluded that the formal dehydrogenation mechanism in which a hydride ion is transferred to NAD (or NADP) and a proton is eliminated to the medium is not applicable to the transformation of 5α-cholest-7-en-3β-ol (**5**) to cholesta-5,7-dien-3β-ol (**6**).

Several years ago two mechanisms [20, 21] were suggested which could explain our results. It was proposed that the molecular oxygen required for the conversion of (**5**) to (**6**) is the hydrogen acceptor and is reduced in the process to hydrogen peroxide [20]. We have tested this hypothesis and were unable to detect the generation of hydrogen peroxide in the dehydrogenation reaction [22]. The sensitivity of the analytical procedure employed by us exceeded fourfold the amount of hydrogen peroxide which could

have been produced [22]. Slaytor and Bloch have shown that 5α, 6α and 6β-hydroxylated intermediates are not involved in the desaturation process [21].

It should be pointed out that the results reported in this paper still do not preclude the possibility that *enzyme bound NAD* (or NADP) acts as the hydride acceptor. For example, the NADH (or NADPH) generated during the reaction could remain attached to the enzyme and then be rapidly oxidized. This could account for the presence of the tritium abstracted from the 5α and 6α positions in the water of the medium. The results of Scallen *et al.* [10, 16] and those reported in this paper indicated that the microsomal rat liver acetone powder is very likely depleted of pyridine nucleotides. The fact that the process requires exogenous nucleotides could possibly be rationalized by the need to replenish these cofactors in the enzyme preparation. This could imply that, in order for the reaction to proceed, a portion of the added nucleotide(s) must first become bound to the enzyme.

Alternatively, a mechanism similar to that of the catecholase mediated oxidation of *o*-diphenols may be operating in the 5(6)-desaturation [22]. We have now obtained evidence for the participation of microsomal cytochrome b_5 in the process [23]. At present, the mechanism of the removal of the 5α and 6α hydrogens atoms of (**5**) is still not clear and requires further studies, preferably with a purified enzyme preparation.

Acknowledgement—This work was supported by NIH grant AM 12156.

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