The Biological Conversion of 7-Dehydrocholesterol into Cholesterol and Comments on the Reduction of Double Bonds

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It is shown that the 7-dehydrocholesterol reductase-catalysed conversion of 7-dehydrocholesterol into cholesterol (II), with a 105 000g microsomal pellet of rat liver in the presence of $[4\text{-}^3\text{H}_2]\text{NADPH}$, results in the transfer of radioactivity to the 7α -position of cholesterol. When the conversion is carried out in the presence of tritiated water the label is introduced exclusively at the 8β -position. However, when the conversion of 7-dehydrocholesterol into cholesterol is performed with a 500g supernatant of rat liver homogenate the radioactivity is incorporated at both the 7α - and the 8β -position. Evidence is provided for the presence of an enzyme system in the 500g supernatant that catalyses an equilibration of hydrogen atoms between those at the 4-position of NADPH and those of water. The work with stereospecifically labelled cofactors shows that both the equilibrating system and the 7-dehydrocholesterol reductase utilize the 4B-hydrogen atom of NADPH. In the light of these results a mechanism for the reduction of carbon-carbon double bonds is discussed.

The rigid stereochemistry of the steroid nucleus has in the past been extensively utilized in the discovery of a number of fundamental principles of organic chemistry. One of the best known examples of this is the Barton theory of conformational analysis. We hoped that the steroid nucleus, where it is possible to define unambiguously the conformation at each carbon atom, might offer similar advantages in the study of biochemical mechanisms.

Recent work has shown that the final stage in cholesterol biosynthesis is the 7-dehydrocholesterol reductase-catalysed conversion of a 5,7-diene system (partial structure I) into a 5-ene system (partial structure II) (Dempsey, 1965; Dvornik, Kraml & Bagli, 1966; Akhtar, Wilton & Munday, 1966b).

In this paper we describe the complete mechanism of this transformation, 7-dehydrocholesterol $\rightarrow$ cholesterol, and make generalized proposals about the reduction of carbon-carbon double bonds in biological systems. A preliminary account of this work has been published (Wilton, Akhtar & Munday, 1966).

RESULTS AND DISCUSSION

Conversion of 7-dehydrocholesterol into cholesterol in the presence of $[4\text{-}^3\text{H}_2]\text{NADPH}$. The requirement for NADPH in the enzymic conversion of 7-dehydrocholesterol (I) into cholesterol (II) has been established by Kandutsch (1962) and Dempsey

(1965). In the present work, when the conversion (I) $\rightarrow$ (II) was carried out with a 500g cell-free supernatant of rat liver in the presence of $[4\text{-}^3\text{H}_2]\text{NADPH}$, the derived cholesterol was radioactive, thus demonstrating a direct transfer of tritium from the 4-position of NADPH to the steroid nucleus.

The position of tritium in cholesterol was established by the following degradation. Cholesterol was acetylated to yield the corresponding acetate (III), which on treatment with nitric acid and sodium nitrite gave the nitro compound (IV). On being treated with zinc and acetic acid the nitro compound (IV) gave the 6-oxo compound (V); in the conversion no significant radioactivity was lost. However, on equilibration with methanolic 5% potassium hydroxide the 6-oxo compound (V) lost 93% of the radioactivity of the precursor cholesterol (Expt. 1, Table 1). This amount of radioactivity (93%) must therefore be located at C-7 of the biosynthetic cholesterol because, as is shown in the Experimental section, under the equilibrating conditions employed the 7-hydrogen atoms of the ketone (V) are freely exchanged with the medium.

The stereochemistry of the hydrogen at C-7 was established by the conversion of cholesterol into the 5α -hydroperoxide (VI), when again about 92% of the radioactivity was lost (Expt. 1, Table 1). It is known that in the conversion of cholesterol (II) into 5α -hydroperoxide (VI) it is the 7α -hydrogen

atom that is stereospecifically lost (Nickon & Bagli, 1959, 1961). These experiments demonstrate that, in the enzymic formation of cholesterol (II) from 7-dehydrocholesterol (I), the 7 α -hydrogen atom of the former is derived from the 4-position of NADPH.

Conversion of 7-dehydrocholesterol into cholesterol in the presence of tritiated water. To determine the origin of the second hydrogen atom the conversion (I)→(II) was carried out in the presence of 500g cell-free supernatant of rat liver homogenate, tritiated water and non-radioactive NADPH. The resulting cholesterol was degraded by the sequence cholesterol → cholesterol acetate (III) → 6-nitro compound (IV) → 6-oxo compound (V). The 6-oxo compound (V), on treatment with methanolic 5% KOH, lost 75% of the label of the parent cholesterol, thus demonstrating that this amount of radioactivity was located at the 7-position (Expt. 2, Table 1). The alternative degradation of this

cholesterol to the 5 α -hydroperoxide (VI) showed that once again all the radioactivity at C-7 was located at the 7 α -position (Expt. 2, Table 1).

We have already shown above that in the biosynthesis of cholesterol from 7-dehydrocholesterol the 7 α -hydrogen atom of the former is derived directly from the 4-position of NADPH. The presence of substantial radioactivity of the 7 α -position in cholesterol biosynthesis in the presence of tritiated water therefore remained to be explained. This radioactivity has been attributed to the presence of an enzyme system in the rat liver homogenate that catalyses an equilibration of hydrogen atoms between those at the 4-position of NADPH and those of water, as suggested by equations (1), (2) and (3) (Popják, Goodman, Cornforth, Cornforth & Ryhage, 1961).

Table 1. *Degradation of cholesterol biosynthesized from 7-dehydrocholesterol in the presence of either [4-³H₂]-NADPH or tritiated water*

Expt. 1. Samples (10ml.) of a 500g supernatant of rat liver homogenate were incubated with 2 μ moles of [4-³H₂]NADPH (900000 counts/min./ μ mole) and 300 μ g. of 7-dehydrocholesterol for 3 hr. under anaerobic conditions. The cholesterol isolated contained 2500 counts/min. compared with 200 counts/min. for a control flask containing no substrate. Expt. 2. Samples (10ml.) of a 500g supernatant of rat-liver homogenate were incubated with 2 μ moles of NADPH, 15 μ moles of glucose 6-phosphate, 300 μ g. of 7-dehydrocholesterol and 200 mc of tritiated water for 3 hr. under anaerobic conditions. The cholesterol isolated contained 23700 counts/min. compared with 360 counts/min. for a control flask containing no substrate. Expt. 3. Samples (5ml.) of 105000g microsomes were incubated with 0.8 μ mole of [4-³H₂]NADPH (900000 counts/min./ μ mole) and 1 mg. of 7-dehydrocholesterol for 3 hr. under anaerobic conditions. The cholesterol isolated contained 5380 counts/min. compared with 89 counts/min. for a control flask containing no substrate. Expt. 4. Samples (5ml.) of 105000g microsomes were incubated with 1 μ mole of NADP, 7 μ moles of glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, 1 mg. of 7-dehydrocholesterol and 50 mc of tritiated water for 3 hr. under anaerobic conditions. The cholesterol isolated contained 5890 counts/min. compared with 100 counts/min. for a control flask containing no substrate.

Expt. no.	System used for incubation	Radioactivity of compounds (counts/min./m-mole)					Radioactivity (%)	
		Cholesterol (II)	6-Nitro compd. (IV)	6-Oxo compd. (V)	6-Oxo compd. + base (V)	5 α -Hydroperoxide (VI)	At C-7	At C-8
1	500g cell-free supernatant + [4- ³ H ₂]NADPH	(a) 5800	—	5120†	440	—	93	—
		(b) 5650	—	—	—	473	96*	—
2	500g cell-free supernatant + tritiated water	11760	—	—	2900	—	75	25
		9050	8800	7540†	2320	2520	74	26
3	105000g microsomes + [4- ³ H ₂]NADPH	14977	—	—	—	846	98*	—
4	105000g microsomes + tritiated water	6405	—	—	—	6313	1	99

* Corrected for 96% stereospecificity for the formation of the 5 α -hydroperoxide (VI) (Nickon & Bagli, 1959).

† Partial loss due to equilibration under acid conditions.

The radioactivity at C-7 in cholesterol biosynthesized from 7-dehydrocholesterol in the presence of tritiated water could have originated only from NADPH formed by the equilibration reaction (3). The equilibration reaction (3) does not affect the main conclusion based on the [4-³H₂]-NADPH experiments because any equilibration of the label in these experiments would hardly affect the net radioactivity of the hydrogen atoms of the water. The equilibration reaction, however, decreases the incorporation of label from [4-³H₂]-NADPH into cholesterol.

The remaining 25% of the radioactivity of cholesterol biosynthesized in the presence of tritiated water has been shown to be at the 8 β -position of cholesterol by using the degradative procedure described by Akhtar *et al.* (1966b).

We therefore conclude that in the conversion of 7-dehydrocholesterol into cholesterol the 7 α - and the 8 β -hydrogen atoms are derived from NADPH and a suitable proton source respectively. The overall addition is *trans*-diaxial as shown in structure

(VII) (Fig. 1). This *trans* addition of hydrogen in the reaction (I) $\rightarrow$ (II) may be compared with the *cis* elimination of hydrogen that is involved in the biosynthesis of 7-dehydrocholesterol from its precursor, Δ^7 -cholestenol (Akhtar & Marsh, 1967; Dewhurst & Akhtar, 1967; Paliokas & Schroepfer, 1967).

Determination of the stereospecificity of the hydrogen atom transferred from NADPH. The pyridine nucleotide-linked enzymes so far studied have all shown an absolute stereospecificity with respect to the hydrogen atom at the 4-position of the pyridine nucleotide. The stereospecificity of the 7-dehydrocholesterol reductase system was determined by incubating 7-dehydrocholesterol in the presence of stereospecifically labelled NADPH. The work of Popják *et al.* (1961) indicates that the equilibrating enzyme system is restricted to the soluble fraction (105000g supernatant) of rat liver homogenate. Therefore by using the 105000g microsomes, which contain a significant proportion of the total 7-dehydrocholesterol reductase activity of the liver (Kandutsch, 1962; M. Akhtar & S. J. M. Skinner, unpublished work) it was hoped that a reasonable incorporation from [4-³H₂]NADPH into

Table 2. *Anaerobic incubation of 7-dehydrocholesterol with 105 000g microsomes from rat liver in the presence of [4A-³H]NADPH, [4B-³H]NADPH or [4-³H]NADP*

Samples (5ml.) were incubated with 2 μ moles of radioactive cofactor and 1mg. of 6-dehydrocholesterol for 2½ hr.

Expt. no.	Cofactor added	Sp. radio-activity (counts/min./ μ mole)	Total radio-activity in cholesterol (counts/min.)
1	[4A- ³ H]NADPH	300 000	761
2	[4B- ³ H]NADPH	300 000	5610
3	[4- ³ H]NADP	300 000	32

cholesterol might be obtained. Indeed when 7-dehydrocholesterol was incubated with 105 000g microsomes and [4-³H₂]NADPH (chemically prepared) a satisfactory incorporation of radioactivity into cholesterol was obtained (Expt. 3, Table 1). The degradation of this radioactive cholesterol to the 5 α -hydroperoxide (VI) resulted in the loss of 94% of the radioactivity (Expt. 3, Table 1), thus confirming that the 7 α -hydrogen atom of cholesterol is derived from the 4-position of NADPH.

[4-³H]NADP was prepared from [4-³H₂]NADPH (chemically prepared) by oxidation with glutathione reductase. This [4-³H]NADP was reduced with either a glucose 6-phosphate dehydrogenase system to give [4A-³H]NADPH or with an isocitrate dehydrogenase system to give [4B-³H]NADPH. The results of the incubation of stereospecifically labelled NADPH with 7-dehydrocholesterol and 105 000g microsomes (Table 2) show that there is a good incorporation of radioactivity from [4B-³H]-NADPH into cholesterol (Table 2).

These results establish that the hydrogen atom transferred to the cholesterol in the conversion of 7-dehydrocholesterol into cholesterol originates from the 4B-position of NADPH. The B stereospecificity agrees with the observations of Popják, Schroeffer & Cornforth (1962), i.e. that the hydrogen atom derived from NADPH in the condensation of farnesyl pyrophosphates to give squalene is from the B side and that this condensing system is also subject to equilibration (Popják *et al.* 1961).

Incubation of 7-dehydrocholesterol with 105 000g microsomes in the presence of tritiated water. If the microsomal 7-dehydrocholesterol reductase system is devoid of equilibrating enzyme activity then the conversion of 7-dehydrocholesterol into cholesterol by the microsomes, in the presence of tritiated water, should result in no incorporation of radioactivity into the 7 α -position of cholesterol. When the radioactive cholesterol obtained from such an incubation was photo-oxidized to the 5 α -hydroperoxide (VI), this transformation was accompanied

Table 3. *Anaerobic incubation of [4-³H₂]NADPH with a 500g cell-free supernatant of rat-liver homogenate and the appearance of radioactivity in the medium*

Expt. no.	Cofactor added	Concn. of cofactor in the medium (μ mole/ml.)	% of [4- ³ H ₂]NADPH radioactivity liberated after 5 min.
1	[4- ³ H ₂]NADPH	0.46	51
2	[4- ³ H ₂]NADPH	0.52	57
3	[4A- ³ H]NADPH	0.45	27
4	[4B- ³ H]NADPH	0.45	91

by the complete retention of radioactivity (Expt. 4, Table 1), thus showing that no radioactivity is incorporated into the 7 α -position of cholesterol.

Studies on the equilibrating enzyme system. A more direct evidence for the presence of an equilibrating enzyme system in the 500g cell-free supernatant of rat liver was provided by the following experiments. When [4-³H₂]NADPH (chemically prepared) was incubated under anaerobic conditions about 50% of the available radioactivity was recovered in the medium after incubation for 5 min. (Expts. 1 and 2, Table 3). The radioactivity of the medium increased only slowly during longer incubations. To determine which side of the NADPH was being equilibrated specifically labelled [4A-³H]NADPH and [4B-³H]NADPH were incubated with a 500g cell-free supernatant under identical conditions and the activity of the medium was determined after 5 min. The results (Table 3) show that the [4B-³H]NADPH loses over 90% of its available radioactivity after 5 min. incubation whereas with the [4A-³H]NADPH the loss is only 27%. These results establish that in the 500g cell-free supernatant of the rat-liver homogenate there is an enzyme system present that catalyses a rapid loss of activity from the B side of NADPH.

Mechanism and general considerations. Most biological reactions in which pyridine nucleotides act as cofactors involve a formal addition or removal of an element of hydrogen. If we consider

$$\begin{array}{c} | \\ \text{the reduction of a hypothetical compound } C=Y \\ | \end{array}$$

$$\begin{array}{c} | \\ \text{to } H-C-Y-H \text{ then there are at least three} \\ | \end{array}$$
mechanisms through which this transformation may occur.

In mechanism 1 the crucial event is the nucleophilic attack by the reducing agent, resulting in the formation of a negatively charged species (α) which is then neutralized by a proton addition.

Mechanism 1.

Mechanism 2.

Mechanism 3.

In mechanism 2 the reaction is initiated by the electrophilic addition of a proton to $\text{C}=\text{Y}$, giving

the carbonium ion intermediate (b) which is then neutralized by the delivery of a hydride ion from NAD(P)H to furnish the product.

Mechanism 3 involves a concerted addition of H^+ and H^- .

Chemical considerations would permit any one of these three mechanisms for the reduction of carbonyl and imino compounds and, indeed, from time to time all these mechanisms, or their equivalents, have been considered for the alcohol dehydrogenase reaction. However, convincing experimental evidence to verify them has not been forthcoming. Steroid hormones containing the 4-en-3-one systems (partial structure VIII) are readily metabolized in the liver to the corresponding dihydro derivatives (partial structure IX). It is now well established that NADPH-linked enzymes are involved in these reduction reactions. Again, although all three mechanisms are possible, Ringold (1964) has preferred a mechanism of type 1 (see above) for the conversion (VIII) $\rightarrow$ (IX).

In the light of the experimental data presented in this paper we shall now attempt to consider the mechanism for the reduction of the carbon-carbon double bond in the conversion of 7-dehydrocholesterol into cholesterol. It has been shown above that in the conversion the 7- and the 8β -hydrogen atoms of the biosynthetic cholesterol are derived from the 4-position of NADPH and a proton source

respectively. When the conversion is carried out in tritiated water and in the presence of the equilibrating enzyme system the amount of radioactivity incorporated at the 7α -position is three times as much as that incorporated at the 8β -position. This would mean that a larger isotope effect is associated with the formation of the proton-derived 8β -hydrogen atom than with the formation of the NADPH-derived 7α -hydrogen atom. The relatively large isotope effect associated with the 8β -hydrogen atom suggests that the formation of this bond may be involved in the rate-determining step of the reaction. This would eliminate mechanism 1 and make mechanism 3 highly unlikely. It is therefore suggested that a mechanism of type 2 is involved in the conversion of 7-dehydrocholesterol into cholesterol. If this mechanistic suggestion is accepted it then follows that the chemical factors that control the addition of hydrogen to the C-7-C-8 double bond of (I) are the same as those responsible for the Markownikoff addition of H^+X^- to olefinic linkages in chemical reactions. That is to say in the addition of H^+X^- the proton adds to the double bond in such a way that the most stable carbonium ion intermediate is formed.

The reduction of carbon-carbon double bonds also occurs in the elaboration of the isoprenoid side chains of C_{27} steroids (Avigan & Steinberg, 1961; Avigan, Goodman & Steinberg, 1963; Wilton *et al.* 1966), vitamin E and vitamin K (Goodwin, 1963) and phytol (Wellburn, Stone & Hemming, 1966) and in the biosynthesis of ergosterol and other methyl- and ethyl-substituted steroids (Akhtar, Parvez & Hunt, 1966a; Akhtar, Hunt & Parvez, 1967). The present work prompts us to forecast that mechanism 2 may also be involved in the reductive steps of these biosyntheses. The prediction would require that for an isolated unsymmetrical double bond the reduction may operate through the addition of an enzyme-bound

proton to the less-substituted carbon atom and the addition of a hydride ion, or its equivalent, to the more substituted carbon atom.

EXPERIMENTAL

Materials. 7-Dehydro[19-³H]cholesterol (71000 counts/min./μmole) was prepared from [19-³H]cholesterol (Akhtar & Gibbons, 1965a,b) and was shown to contain less than 4% of cholesterol as contaminant. This material was used throughout the investigation for checking the yield in the biological conversion of 7-dehydrocholesterol into cholesterol. 7-Dehydrocholesterol acetate, haematoporphyrin IX dihydrochloride, Tween 80 and cholesterol were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. The 7-dehydrocholesterol acetate was hydrolysed and crystallized before use. The cholesterol was recrystallized three times from acetone before use. NADP, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, GSSG and glutathione reductase were obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. DL-Isocitric acid and isocitrate dehydrogenase were obtained from Calbiochem Ltd., London, W. 1.

Silica gel for thin-layer chromatography was obtained from E. Merck A.-G., Darmstadt, Germany. All solvents were normal laboratory grade. All other chemicals mentioned were obtained from British Drug Houses Ltd., Poole, Dorset. Scintillation fluids NE213 and NE220 were supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh. 2-(4-*tert.*-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole, was obtained from Ciba (A.R.L.) Ltd., Duxford, Cambs. Tritiated water was obtained from The Radiochemical Centre, Amersham, Bucks.

Preparation of homogenates and incubation procedure. Male Wistar albino rats (200–300g.) were used. They were killed by cervical fracture and the excised livers were homogenized in cold 0.1M-sodium phosphate buffer, pH 7.3, containing 30mM-nicotinamide and 4mM-MgCl₂ (1.25ml./g. of liver). A loose-fitting piston-barrel-type homogenizer was used (Bucher, 1953). The 500g cell-free supernatant was prepared by centrifuging the homogenate at 500g for 15 min. The supernatant was decanted and used. The 105000g microsomes were prepared by homogenizing the liver in 0.25M-sucrose (2ml./g. of liver) and then centrifuging the homogenate at 10000g for 15 min. The supernatant was decanted and centrifuged at 105000g for 1 hr. The pellet was resuspended in the 0.1M-sodium phosphate buffer described above (1ml./g. of liver) and centrifuged again at 120000g for 45 min. The pellet was resuspended as before and used. All the above preparations were carried out at 0–5°. Incubations were performed in 50ml. or 100ml. Erlenmeyer flasks at 37° with gentle shaking.

In all experiments with the 500g cell-free supernatant preparation the 7-dehydrocholesterol (1mg.) was dissolved in propylene glycol (1ml.) by rapid heating at 100°. A portion (0.2–0.5ml.) of the solution was used/10ml. of cell-free supernatant.

In the experiments with 105000g microsomes the 7-dehydrocholesterol (1mg.) was dissolved in a minimal quantity of acetone and Tween 80 (25mg.) was added. After the acetone had been evaporated off with N₂ the solid was resuspended in 1ml. of water to give an emulsion.

Anaerobic conditions were achieved by using CO₂, which was generated from solid CO₂.

Isolation and purification of cholesterol. All incubations were terminated by the addition of methanolic 10% KOH (10ml.). Cholesterol (100mg.) was added in acetone (10ml.) and the mixture was saponified at 60° for 5 min. The cholesterol was extracted with 4 × 25ml. of light petroleum (b.p. 40–60°)-diethyl ether (1:1, v/v). The combined extracts were washed with saturated NaHCO₃ solution, dried with Na₂SO₄ and evaporated to dryness. The residue was spotted on a silica-gel plate (20cm. × 9cm., 2mm.-thick layer), which was developed in acetone-light petroleum (b.p. 60–80°) (1:3, v/v), and then located by viewing in ordinary light. The cholesterol band was extracted with ether and purified via the dibromide, as follows. Cholesterol (100mg.) was dissolved in diethyl ether (1.5ml.) and acetic acid (0.5ml.) was added. The solution was cooled in ice and Br₂ (0.1ml.) was added dropwise. After 15 min. acetic acid (2ml.) was added and, after crystallization was complete, the dibromide was filtered off and washed with cold methanol. The cholesterol dibromide was crystallized from diethyl ether-methanol; the yield was 80 mg. The dibromide was dissolved in 10ml. of diethyl ether, and 2g. of zinc and a trace of acetic acid were added. The solution was stirred vigorously at room temperature for 2 hr. The cholesterol was worked up in the usual way and normally diluted before crystallization.

Measurement of radioactivity. Radioactive samples (10–30mg.) were weighed out directly into glass vials and counted in an Isotope Developments Ltd. no. 2022 liquid-scintillation counter at about 20% efficiency or in a Beckman 200 CPM instrument at about 50% counting efficiency. Samples were adjusted for quenching by internal standardization. For experiments associated with the 5α-hydroperoxy compound (VI) the dioxan-based NE220 was used; otherwise sterols were dissolved in NE213 or in 2-(4-*tert.*-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole in toluene (8g./l.).

Conversion of cholesterol into 3β-acetoxycholestan-6-one. (a) 3β-Acetoxy-6-nitrocholest-5-ene (6-nitro compound; IV). The reaction of cholesteryl acetate (130mg.) in conc. HNO₃ (4ml.) with NaNO₂ (100mg.) for 27 min. by the method described by Akhtar & Marsh (1967) gave the nitro compound (IV) (90mg.).

(b) 3β-Acetoxycholestan-6-one (6-oxo compound; V). A solution of 3β-acetoxy-6-nitrocholest-5-ene (90mg.) in acetic acid (6.2ml.) and water (0.7ml.) was treated with zinc (1.0g.) and the reaction mixture was vigorously stirred at 80° for 4 hr. Zinc was then removed by filtration and the filtrate extracted with ether. The organic layer was washed with saturated NaHCO₃ solution, dried with Na₂SO₄ and evaporated to dryness. Crystallization from methanol gave the 6-oxo compound (V) (30mg.).

(c) 3β-Acetoxy-[7-³H]cholest-6-one. 3β-Acetoxy-7α-bromocholestan-6-one was debrominated in the presence of tritiated water to give 3β-acetoxy-[7-³H]cholest-6-one. This ketone, when treated overnight with methanolic 5% KOH, lost 99% of the original radioactivity, thus showing that this method is a valid procedure for determining the radioactivity at C-7 of cholesterol.

Conversion of cholesterol into cholest-6-en-5α-hydroperoxy-3β-ol (5α-hydroperoxy compound; VI). Cholesterol (100mg.) and haematoporphyrin IX dihydrochloride (15mg.) were dissolved in pyridine (20ml.) in a Pyrex boiling tube. A

'cold finger' and 'oxygen bleed' were inserted into the solution, which was irradiated externally for 24 hr. by a normal 150 w frosted-glass bulb placed 2 in. away. The apparatus was surrounded by aluminium foil to act as a light-reflector.

The product was extracted into ether (25 ml.) and the extract was washed with 0.2 N-HCl (4 × 25 ml.) to remove all trace of pyridine. More ether was added and the extract washed with saturated NaHCO₃ solution, then dried with Na₂SO₄ and evaporated to dryness. Crystallization from ether-methanol gave the 5 α -hydroperoxy compound (35 mg.).

Preparation of [4-³H₂]NADPH. NADP (50 mg.) and NaHCO₃ (34 mg.) were placed in the main compartment of a Warburg manometric flask and Na₂S₂O₄ (24 mg.) was placed in the side arm. Tritiated water (1 ml., normally 1 c) was added to the main compartment and, after it had been put in a bath at 25°, the apparatus was flushed out with CO₂. The Na₂S₂O₄ was washed into the main compartment to give a yellow solution. When there was no more CO₂ evolution (about 30 min.) the contents of the flask were diluted with a further 1 ml. of water and freeze-dried. The freeze-dried material was dissolved in water (2 ml.) and acetone (30 ml.) at -15° was added to give a white precipitate. After 45 min. at -15° the solution was centrifuged and the acetone was decanted off. The sediment was allowed to dry in the air, dissolved in 10 mm-KHCO₃ (5 ml.) and freeze-dried. The final product (about 900 000 counts/min./ μ mole) was 80% pure.

Preparation of [4-³H]NADP. The method used involved the enzymic oxidation of [4-³H₂]NADPH with a glutathione reductase system. To [4-³H₂]NADPH (13 μ moles, 9.7 mg.) dissolved in 10 ml. of 0.1 M-sodium phosphate buffer, pH 7.2, was added GSSG (13 μ moles, 8.0 mg.) and glutathione reductase (3.5 units). When the oxidation, which was followed by the change in extinction at 340 m μ , was complete the system was freeze-dried.

Preparation of [4A-³H]NADPH. To [4-³H]NADP (1.3 μ moles, 0.97 mg.) dissolved in 1.0 ml. of 0.1 M-sodium phosphate buffer, pH 7.2, was added glucose 6-phosphate (5.0 μ moles, 1.5 mg.) and glucose 6-phosphate dehydrogenase (1.4 units). The [4A-³H]NADPH produced was 80% pure when measured spectrophotometrically.

Preparation of [4B-³H]NADPH. To [4-³H]NADP (1.3 μ moles, 0.97 mg.) dissolved in 1.0 ml. of 0.1 M-sodium phosphate buffer, pH 7.2, was added DL-isocitric acid (10 μ moles, 2.0 mg.) and isocitrate dehydrogenase (1.8 units). The [4B-³H]NADPH produced was 80% pure when measured spectrophotometrically.

Distribution of radioactivity at C-4 of tritiated NADPH. This was determined by oxidizing [4-³H₂]NADPH (13 750 counts/min.) with the B-specific glutathione reductase. The incubation mixture was freeze-dried and the condensate counted. The radioactivity of the condensate (4000 counts/min.) corresponded to the radioactivity on the B side of the [4-³H₂]NADPH. The resulting [4-³H]NADP was reduced with the A-specific isocitrate dehydrogenase to give [4B-³H]NADPH. This was again oxidized with glutathione reductase and the reaction mixture freeze-dried and the condensate counted (6700 counts/min.). This radioactivity corresponded to the A side of the original [4-³H₂]NADPH. Therefore the radioactive purity of the [4-³H₂]NADPH was 78% (i.e. $\frac{4000 + 6700}{13750} \times 100\%$), which

corresponded to its chemical purity. It is noteworthy that this method of degradation of the [4-³H₂]NADPH demonstrates the unequal distribution of label between the A and B sides (A side 62.5%, B side 37.5%). Similar observations have been recorded for NADH reduced by the Na₂S₂O₄ method (Fisher, Conn, Vennesland & Westheimer, 1953; Pullman, San Pietro & Colowick, 1954).

Determination of radioactivity at C-7 and C-8 of cholesterol biosynthesized from 7-dehydrocholesterol with a 500g supernatant of rat liver in the presence of tritiated water. Cholesterol (2.0 g., 13 650 counts/min./m-mole) in acetic anhydride (8 ml.) was boiled under reflux for 1½ hr. After being diluted with acetic acid (24 ml.) the reaction mixture was treated with sodium acetate (1.6 g.) and CrO₃ (2.0 g.). This mixture was stirred under an atmosphere of purified N₂ for 5 hr. at 50–55°. Dropwise addition of water (5 ml.) gave 3 β -acetoxycholest-5-en-7-one (500 mg., 3410 counts/min./m-mole). This ketone (500 mg.) in diethyl ether (25 ml.) was boiled with LiAlH₄ (200–300 mg.) for 1½ hr. and the resulting diol, cholest-5-ene-3 β ,7 β -diol, was treated with benzoyl chloride (1.5 ml.) in pyridine (10 ml.) to yield cholest-5-ene-3 β ,7 β -diol dibenzoate (340 mg.) (Fieser, 1953). This dibenzoate was dissolved in dimethylaniline (10 ml.) and converted into 7-dehydrocholesterol benzoate (130 mg., 196 counts/min./m-mole) by the method of Haslewood (1938). This degradation therefore proves that the biosynthetic cholesterol contains 75% of its radioactivity at C-7 and the remaining 25% at C-8.

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