The Stereochemistry of Hydrogen Transfer from Reduced Nicotinamide-Adenine Dinucleotide Phosphate in the Reduction of Ethylenic Linkages during Cholesterol Biosynthesis

By D. C. WILTON, I. A. WATKINSON AND M. AKHTAR

Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH, U.K.

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It is shown that during the saturation of steroid carbon-carbon double bonds at $\Delta^{24,25}$ and $\Delta^{14,15}$ the 'hydride ion' originates from the 4B side of the NADPH.

In the later stages of cholesterol biosynthesis a number of carbon-carbon double bonds are introduced into the sterol molecule and subsequently saturated by a series of NADPH-linked sterol reductases. These reductive transformations include the saturation of double bonds at $\Delta^{24,25}$ (I $\rightarrow$ III), $\Delta^{7,8}$ (II $\rightarrow$ III) and $\Delta^{14,15}$ (IV $\rightarrow$ VI). During an investigation on these reactions it has been established that the mechanism of these carbon-carbon double bond reductions is typical of a *trans*-directed Markownikoff addition process (Wilton, Munday, Skinner & Akhtar, 1968; Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969*a*; Akhtar, Rahimtula & Wilton, 1969*b*; Akhtar, Munday, Rahimtula, Watkinson & Wilton, 1969*c*; Caspi, Galli Kienle, Varma & Mulheirn, 1970). It was shown in each case that the orientation of saturation was consistent with the initial addition of a proton such that the most stable carbonium ion intermediate was generated and that this carbonium ion was subsequently neutralized by the delivery of a hydride ion from the NADPH. Thus the proton is incorporated at the 8β -position in the $\Delta^{7,8}$ -double-bond reduction, at the 15β -position in the $\Delta^{14,15}$ -double-bond reduction and at the 24-*pro-S*-position in the $\Delta^{24,25}$ -double-bond reduction. The 'hydride ion' from NADPH is then added into the 7α -, the 14α - and the 25-position respectively. This stereochemical information is summarized in Scheme 1.

The purpose of the present paper is to extend this information to include observations on the stereochemical origin of the hydrogen transferred from the NADPH during each reduction. We have previously reported that with the sterol $\Delta^{7,8}$ -reductase this hydrogen originates from the 4B side of the NADPH (Wilton *et al.* 1968).

RESULTS AND DISCUSSION

Table 1 describes the results of incubation of various sterols (I, IV, V, IX and X) with rat liver

microsomes in the presence of [4A- 3 H]- and [4B- 3 H]-NADPH under anaerobic conditions. It is clear from the total radioactivity incorporated into the reduced product that in each case the tritium originates predominantly from the 4B side of the cofactor. It is of note that all carbon-carbon double-bond reductions involving the transfer of a hydride ion to the α (axial) side of the steroid nucleus involves the 4B side of the NADPH (see above and also the steroid 5α -reductase; Wilton & Ringold, 1968; Bjorkhem, 1969). On the other hand the single reported case of hydride ion transfer to the β (equatorial) side of the steroid nucleus during carbon-carbon double bond reduction, with the steroid 5β reductase, involves the 4A side of the NADPH (Berseus & Bjorkhem, 1967). This apparent stereochemical correlation may well either have a hitherto unappreciated mechanistic implication or reflect a common evolutionary pathway for the development of these steroid enzymes.

EXPERIMENTAL

Materials. [4A- 3 H]- and [4B- 3 H]-NADPH (10^6 c.p.m./ μ mol) were prepared by the method of Wilton *et al.* (1968). Cholesta-5,24-dien-3 β -ol (I) was prepared from 3 β -acetoxy-26-norcholest-5-en-25-one by the method of Dauben & Bradlow (1950). 4,4'-Dimethylcholesta-8,14-dien-3 β -ol (IV) was prepared by the method of Akhtar, Watkinson, Rahimtula, Wilton & Munday (1969*e*). Cholesta-8,14-dien-3 β -ol (V) was prepared by the method of Fieser & Ourisson (1953). Cholesta-7,14-dien-3 β -ol (X) was prepared by the method of Akhtar, Rahimtula, Watkinson, Wilton & Munday (1969*d*).

Preparation of 4,4'-dimethylcholesta-7,14-dien-3 β -ol (IX). 4,3 β -Acetoxy-4'-dimethylcholesta-5,7-diene (20 mg) was dissolved in chloroform (10 ml) at -30°C and dry HCl gas was bubbled through the mixture for 2 h. The resulting mixture was poured into rapidly stirring ice-cold saturated NaHCO₃ solution and extracted with chloroform. The chloroform extract was washed with water and saturated NaCl, then dried (Na₂SO₄) and evaporated to dryness. The residue was spotted on a freshly prepared silica gel-10% AgNO₃ plate (20 cm $\times$ 10 cm; 1 mm-thick layer; fluorescent) and developed in benzene-light petroleum (b.p.

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Scheme 1.

60–80°C) (7:3, v/v). The diene band (R_F 0.44) was eluted and crystallized. The material had ϵ 10150 at λ_{max} , 243 nm [Fieser & Fieser (1959) quote ϵ 9900].

Incubation procedure. Microsomes were prepared from male Wistar Albino rats by the method described by Wilton *et al.* (1968). The microsomes (5 ml) were added to 1 mg of the sterol solubilized in 50 mg of Tween 80. After anaerobic conditions had been obtained the radioactive

cofactor (2 μ mol) was added and the mixture was incubated under anaerobic conditions for 90 min. Incubations were terminated by the addition of 5 ml of methanolic 10% (w/v) KOH.

Isolation and purification of products. In Expt. 1 (Table 1) cholesterol (II) was isolated as the dibromide after the addition of non-radioactive cholesterol (100 mg) (Wilton *et al.* 1968). In Expts. 3 and 5 (Table 1) non-

Table 1. Incubation of [4A-³H]- and [4B-³H]NADPH with rat liver microsomes and unlabelled sterols under anaerobic conditions

Expt no.	Sterol incubated	Cofactor incubated	Radioactivity incorporated into product		Double bond reduced
			(c.p.m.)	(%)	
1	Cholesta-5,24-dien-3 β -ol (I)	[4A- ³ H]NADPH	8400	(19)	$\Delta^{24,25}$
		[4B- ³ H]NADPH	43300	(81)	
2	4,4'-Dimethylcholesta-8,14-dien-3 β -ol (IV)	[4A- ³ H]NADPH	7140	(13)	$\Delta^{14,15}$
		[4B- ³ H]NADPH	59200	(87)	
3	Cholesta-8,14-dien-3 β -ol (V)	[4A- ³ H]NADPH	7900	(29)	$\Delta^{14,15}$
		[4B- ³ H]NADPH	19400	(71)	
4	4,4'-Dimethylcholesta-7,14-dien-3 β -ol (IX)	[4A- ³ H]NADPH	16240	(23)	$\Delta^{14,15}$
		[4B- ³ H]NADPH	55000	(77)	
5	Cholesta-7,14-dien-3 β -ol (X)	[4A- ³ H]NADPH	3160	(11)	$\Delta^{14,15}$
		[4B- ³ H]NADPH	28500	(89)	

radioactive cholest-7-en-3 β -ol (VIII) (20 mg) was added after termination of the incubation and the sterols were extracted (Wilton *et al.* 1968). The sterols were acetylated and then separated by t.l.c. under the chromatographic conditions described above. The 3 β -acetoxycholest-7-ene band (R_F 0.7) was eluted and rechromatographed as above and finally crystallized. Under the conditions of the incubation the cholest-8-en-3 β -ol (VII) produced in Expt. 3 (Table 1) will be isomerized to cholest-7-en-3 β -ol (VIII) by the microsomal sterol $\Delta^{8,9}$ - $\Delta^{7,8}$ -isomerase (Gaylor, Delwiche & Swindell, 1966). In Expts. 2 and 4 (Table 1) non-radioactive 4,4'-dimethylcholest-8-en-3 β -ol (VI) (20 mg) was added after termination of the incubation, and this was isolated and purified by the method described above for cholest-7-en-3 β -ol. Owing to the non-availability of 4,4'-dimethylcholest-7-en-3 β -ol (XI) it was necessary to use the 4,4'-dimethylcholest-8-en-3 β -ol (VI) as carrier in Expts. 2 and 4.

Measurement of radioactivity. For details see Akhtar, Rahimtula & Wilton (1969f).

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