The Role of a 5α -Hydroxylated Intermediate in the Formation of the 5,6-Double Bond in Cholesterol Biosynthesis

By KEN ALEXANDER and MUHAMMAD AKHTAR

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

(Received 5 August 1974)

If the biological conversion of cholest-7-en- 3β -ol (I) into cholesterol (IV) occurred through the intermediacy of cholest-7-ene- $3\beta,5\alpha$ -diol (II) then the factor(s) adversely affecting the conversion of the 5α -hydroxy sterol (II) into cholesterol must at least equally adversely affect the formation of cholesterol from cholest-7-en- 3β -ol. By using partial denaturation techniques and dual-labelled precursors it was shown that the enzyme system responsible for the conversion of the 5α -hydroxy sterol (II) into cholesterol denatured faster than that for the corresponding conversion from cholest-7-en- 3β -ol (I).

The penultimate stage in the hepatic biosynthesis of cholesterol involves the introduction of the 5,6-double bond in cholest-7-en- 3β -ol (structure I, Scheme 1) to give the 5,7-diene system of 7-dehydrocholesterol (III), which is then converted into cholesterol (IV) by a NADPH-linked reduction process (Frantz *et al.*, 1959; Dempsey, 1965; Akhtar & Marsh, 1967; Dewhurst & Akhtar, 1967; Wilton *et al.*, 1968; for reviews see Frantz & Schroepfer, 1967; Rees & Goodwin, 1971). Several years ago we investigated the stereochemical and enzymic aspects of the introduction of the double bond and showed

that this process occurs through the overall *cis* removal of the 5α - and 6α -hydrogen atoms (Akhtar & Marsh, 1967; Paliokas & Schroepfer, 1968; Dewhurst & Akhtar, 1967). The stereochemical conclusion was confirmed in subsequent studies by using alternative approaches (Akhtar *et al.*, 1969; Goad *et al.*, 1969). Two basic mechanisms were considered for the origin of the double bond, a hydroxylation–dehydration mechanism and a dehydrogenation mechanism (Scheme 2) (Dewhurst & Akhtar, 1967). The hydroxylation–dehydration mechanism requires that one of the three possible hydroxy steroids may participate as

Scheme 1.

an intermediate in the conversion of (I) into (IV). Work of Slaytor & Bloch (1965) had shown that cholest-7-ene-3 β ,6 β -diol and cholest-7-ene-3 β ,6 α -diol were not involved in the biosynthesis. We synthesized the third potential hydroxylated compound, cholest-7-ene-3 β ,5 α -diol (II), and noted that this compound was converted into cholesterol, but only under aerobic conditions. The cumulative evidence available at that time did not warrant the consideration of the 5 α -hydroxy sterol (II) as a physiological intermediate in the process under consideration.

Topham & Gaylor (1972) described the isolation of an enzyme from yeast which under anaerobic conditions catalysed the conversion of a 5 α -hydroxy-ergost-7-ene derivative (VI) into ergosterol (VIII) by a pathway in which the 3 α -hydrogen of compound (VI) was removed. They postulated the pathway shown in Scheme 3 for the dehydration process and argued that the 5 α -hydroxy compound (VI) could be an intermediary in the desaturation process (V $\rightarrow$ VIII).

We had previously shown that the main stereochemical and enzymic features of the introduction of the 5,6-double bond in ergosterol biosynthesis by the whole cells of *Saccharomyces cerevisiae* were remarkably similar to those for the parallel reaction involved in the hepatic biosynthesis of cholesterol and advocated the participation of an O₂-dependent dehydrogenation mechanism for both the reactions (Akhtar & Parvez, 1968).

The new situation arising from the work of Topham & Gaylor (1972) has necessitated further investigation to evaluate the intermediary role of the 5 α -hydroxy sterols such as compounds (II) and (VI) in the desaturation processes (I $\rightarrow$ (III) and (V) $\rightarrow$ (VIII). In the present paper the relative merits of the two mechanistic alternatives of Scheme 2 have been examined by studying the conversion of cholest-7-en-3 β -ol into cholesterol in rat liver systems.

Materials and Methods

Materials

[26-¹⁴C]Cholesterol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and converted into [26-¹⁴C]cholest-7-en-3 β -ol by the method previously used for the preparation of [4-¹⁴C]cholest-7-en-3 β -ol (Dewhurst & Akhtar, 1967; Dewhurst *et al.*, 1967). These papers also describe the preparation of [3 α -³H]cholest-7-ene-3 β ,5 α -diol. [3 α -³H]Cholest-7-en-3 β -ol was prepared from the corresponding unlabelled material by using the oxidation-reduction sequence used for the synthesis

of [3α - ^3H]cholest-7-ene- 3β , 5β -diol (Dewhurst & Akhtar, 1967).

Methods

Synthesis of [6,7- $^3\text{H}_2$]cholestane- 3β , 5α , 8α -triol 3-acetate. Cholest-7-en- 5α , 8α -epidioxy- 3β -ol 3-acetate (400mg), prepared as described previously (see Addendum to Dewhurst & Akhtar, 1967), was dissolved in ethyl acetate (20ml) and reduced with $^3\text{H}_2$ gas, in the presence of pre-reduced platinum oxide catalyst (40mg). Crystallization from ether-light petroleum (b.p. 40–60°C) gave a highly radioactive product. This product (5mg) on dilution with cholestane- 3β , 5α , 8α -triol 3-acetate (100mg) gave a product containing 4.56×10^7 d.p.m./mmol. Although the stereochemistry of ^3H at C-6 and C-7 is not known, if we assume that addition of hydrogen to the double bond occurs from the α side of the steroid molecule then the labelled atoms will occupy the 6α and 7α positions.

Synthesis of [6,7- ^3H]cholest-7-ene- 3β , 5α -diol. [6,7- $^3\text{H}_2$]cholestane- 3β , 5α , 8α -triol 3-acetate (50mg; specific radioactivity 4.56×10^7 d.p.m./mmol) dissolved in methanol (4ml) was treated with a trace of conc. HCl and the reaction mixture was left at room temperature for 1h. Filtration of the crystallized product gave [6,7- $^3\text{H}_2$]cholest-7-ene- 3β , 5α -diol 3-acetate (20mg; specific radioactivity 3.86×10^7 d.p.m./mmol). The loss of radioactivity from C-7 on dehydration was 15%. Therefore the amount of radioactivity at C-6 and C-7 in the diol is 59:41, assuming equal distribution of label in the parent triol. The hydrolysis of the 3-acetoxy group to give [6,7- $^3\text{H}_2$]cholest-7-ene- 3β , 5α -diol was performed as described previously for the preparation of the unlabelled material (see the Addendum to Dewhurst & Akhtar, 1967).

Since the amount of radioactivity incorporated into cholesterol from [6,7- $^3\text{H}_2$]cholest-7-ene- 3β , 5α -diol was only half of that from [3α - ^3H]cholest-7-ene- 3β , 5α -diol, we have assumed that the ^3H at C-6 of the former compound is removed during the biosynthesis.

Preparation of crude rat liver homogenate. Male Wistar albino rats (100–150g) were killed by cervical dislocation and the excised livers were homogenized in cold (1–4°C) 0.1M-potassium phosphate buffer, pH 7.3, containing 30mM-nicotinamide and 4mM-MgCl₂ (1.25ml/g of liver). This buffer was used throughout. The homogenization consisted of 60 strokes in a loose-fitting piston-barrel-type homogenizer. Cell debris and mitochondria were sedimented by centrifugation at 10000g_{av.} for 15 min at 1–4°C, after which the supernatant was filtered through glass wool to give the crude rat liver homogenate used in the incubations.

Preparation of subcellular fractions. The crude homogenate was centrifuged for 1h at 105000g_{av.}. The 105000g supernatant was then decanted [in one

case (Expt. 1c, Table 2) only the top half of the 105000g supernatant was pipetted off and used for an incubation]. The remaining microsomal pellet was rinsed with cold buffer and resuspended in the same volume of buffer from which it had sedimented. This preparation was used in the 'unwashed microsomal' incubations.

The unwashed microsomal preparation was re-centrifuged for 1h at 105000g. The supernatant was then decanted and the microsomal pellet was rinsed with cold buffer and resuspended in the same volume of buffer from which it had sedimented. This preparation was used in the 'washed microsomal' incubations.

All operations in the above preparations were performed at 1–4°C.

Incubation procedures. Sterols were dissolved in acetone (1ml) containing Tween 80 (25mg). The acetone was then removed with a stream of N₂ and the residue emulsified in water (0.5ml) before the addition of the rat liver preparation. The crude rat liver homogenates were supplemented with glucose 6-phosphate (1.0mM) and GSH (1.0mM). For the microsomal preparations any NADPH generator added contained 4μmol of glucose 6-phosphate and 0.6 unit of glucose 6-phosphate dehydrogenase per μmol of NADP⁺. Incubations were performed in 50ml Erlenmeyer flasks at 37°C with gentle shaking. Aerobic conditions were obtained by incubating in unstoppered flasks and anaerobic conditions were obtained by passing purified N₂ through the incubation flasks. The N₂ was purified from any O₂ contamination by bubbling through a solution prepared from sodium anthraquinone β-sulphonate (2g)+sodium dithionite (15g)+KOH (20g) in water (100ml), and was then dried over CaCl₂.

Isolation and purification of cholesterol as the 5,6-dibromide derivative. Incubations were terminated by the addition of methanolic 10% (w/v) KOH (5ml). Non-radioactive carrier cholesterol (100mg) was then added and the mixture was saponified by heating for 5 min on a steam bath. The cholesterol was extracted with ether (3 × 25ml) and the combined ether extracts were washed with water (3 × 25ml) and saturated NaCl solution (25ml). After drying over anhydrous Na₂SO₄ the solvent was removed under reduced pressure. The solid residue was dissolved in ether (1.5ml) and to this was added 1ml of Fieser's solution [bromine (2ml)+acetic acid (30ml)+sodium acetate (200mg)]. The mixture was cooled in ice for 15 min and the crystallized cholesterol dibromide was washed with cold methanol. Crystallization (three times) from ether-methanol gave pure cholesterol dibromide, yield 70mg m.p. 111–113°C.

Measurement of radioactivity. Radioactive samples were counted in butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in toluene (10ml

portions of 8 g/l solution) at 12°C in an Intertechnique model SP 60 liquid-scintillation counter, programmed to automatic quench correction. Values recorded in d.p.m. were counted at the following efficiencies: ^3H and ^{14}C for double-labelled samples at 43.0 and 77.6% respectively, ^3H and ^{14}C for single-labelled samples at 57.8 and 94.4% respectively.

Results

Status of the 3 α -hydrogen atom in the metabolism of cholest-7-en-3 β -ol and cholest-7-ene-3 β ,5 α -diol

Expts. 1a, 1b and 3 in Table 1 show that when 100 μg of cholest-7-en-3 β -ol (I) was used under aerobic conditions, over 70% was converted into cholesterol; no significant conversion occurred when the incubation was performed under N_2 . In comparison with similar amounts of the 5 α -hydroxy sterol (II), the conversion into cholesterol was only about 15%; once again the latter conversion only occurred in the presence of O_2 (Expts. 2a and 2b, Table 1).

To shed light on the status of the 3 α -hydrogen atom in the conversion (I) $\rightarrow$ (IV) [3α - ^3H ,26- ^{14}C]cholest-7-en-3 β -ol ($^3\text{H}/^{14}\text{C}$ ratio = 1.14) was aerobically converted with rat liver homogenate into cholesterol which had the same $^3\text{H}/^{14}\text{C}$ ratio as the precursor cholest-7-en-3 β -ol (Expt. 3, Table 1). The complete retention of the 3 α - ^3H in the conversion excluded the participation of a pathway of the type shown in Scheme 3 for the biosynthesis.

Next the status of the 3 α -hydrogen atom in the conversion of the 5 α -hydroxy sterol (II) into cholesterol was studied. Ideally for this purpose one needed to perform experiments using doubly labelled precursor of the type described above; however, the unavailability of 5 α -hydroxy sterol (II) labelled with ^{14}C necessitated the adoption of an indirect approach.

[3α - ^3H]Cholest-7-ene-3 β ,5 α -diol and [6,7- $^3\text{H}_2$]cholest-7-ene-3 β ,5 α -diol were separately incubated under aerobic and anaerobic conditions. The failure to detect any anaerobic conversion of the 3 α - ^3H -labelled 5 α -hydroxy sterol was not due to loss of label, since the [6,7- $^3\text{H}_2$]sterol also remained unmetabolized under the same conditions. Both the [3α - ^3H]- and the [6,7- $^3\text{H}_2$]-cholest-7-ene-3 β ,5 α -diols were converted into cholesterol in good yield under aerobic conditions. Thus, in contrast with the mechanism implicated in ergosterol biosynthesis (Scheme 3), the 5 α -hydroxy sterol (II) was only metabolized under aerobic conditions and with the retention of the 3 α -hydrogen atom.

Subcellular fractions of rat liver participating in the metabolism of cholest-7-en-3 β -ol and cholest-7-ene-3 β ,5 α -diol

A systematic study of the subcellular fractions of rat liver was undertaken in an attempt to localize the enzyme systems involved in the metabolism of cholest-7-en-3 β -ol and the 5 α -hydroxy sterol (II). Samples of [26- ^{14}C]cholest-7-en-3 β -ol plus [3α - ^3H]cholest-7-ene-3 β -diol were incubated with either rat liver homogenate, the top half of the 105000g supernatant or washed microsomal fraction supplemented with a NADPH generator (Table 2). Cholest-7-en-3 β -ol was metabolized by the microsomal preparation but not by the top half of the 105000g supernatant. Since the 5 α -hydroxy sterol behaved similarly these results neither support nor exclude an intermediary role for this sterol in the metabolism of cholest-7-en-3 β -ol.

Evidence in support of independent pathways for the metabolism of cholest-7-en-3 β -ol and cholest-7-ene-3 β ,5 α -diol

A comparative study of the time-courses for the metabolism of cholest-7-en-3 β -ol and the 5 α -

Table 1. *Status of 3 α -hydrogen atom during metabolism of cholest-7-en-3 β -ol and cholest-7-ene-3 β ,5 α -diol (II)*

Substrates (100 μg) were incubated with rat liver homogenate (5 ml) in parallel for 90 min. The biosynthesized cholesterol was isolated as the dibromide. Details are described in the Materials and Methods section.

Expt.	Substrate	$^3\text{H}/^{14}\text{C}$ ratio	Gas phase	Biosynthesized cholesterol	
				μg	$^3\text{H}/^{14}\text{C}$ ratio
1a	[26- ^{14}C]Cholest-7-en-3 β -ol (I)	—	Air	70.0	—
1b	[26- ^{14}C]Cholest-7-en-3 β -ol (I)	—	N_2	0	—
2a	[3α - ^3H]5 α -Hydroxysterol (II)	—	Air	15.0	—
2b	[3α - ^3H]5 α -Hydroxy sterol (II)	—	N_2	0.3	—
3	[3α - ^3H ,26- ^{14}C]Cholest-7-en-3 β -ol (I)	1.14	Air	75.4	1.06
4a	[6,7- $^3\text{H}_2$]5 α -Hydroxy sterol (II)	—	Air	15.5*	—
4b	[6,7- $^3\text{H}_2$]5 α -Hydroxy sterol (II)	—	N_2	0.5	—

* Corrected amount of cholesterol formed assuming that the ^3H at C-6 is lost during the metabolism of the 6,7- $^3\text{H}_2$ -labelled 5 α -hydroxy sterol substrate and that the ratio of ^3H at C-6/C-7 is 59:41 (see the Materials and Methods section).

Table 2. Metabolism of cholest-7-en-3β-ol and cholest-7-ene-3β,5α-diol by different subcellular fractions of rat liver

Samples of [3α-³H]cholest-7-ene-3β,5α-diol (100 μg, 1.55 × 10⁵ d.p.m.) plus [26-¹⁴C]cholest-7-en-3β-ol (100 μg, 1.53 × 10⁴ d.p.m.) were aerobically incubated for 30 min with different subcellular fractions (10.0 ml) of rat liver homogenate. In each case both the sterols were incubated in the same flask to minimize differences in experimental conditions for these two substrates. The biosynthesized [3β-³H,26-¹⁴C]cholesterol was isolated as the dibromide. Expt. 1d, carried out with washed microsomal fraction, was supplemented with NADPH generator (1.0 mM-NAD). Since the microsomal preparation in this experiment was resuspended in half the volume of buffer from which it was sedimented the microsomal concentration was double that in the crude homogenate used in Expt. 1a. This presumably explains the improved yield of cholesterol biosynthesized from the 5α-hydroxy sterol in Expt. 1d.

Expt.	Fraction	Cholesterol formed (μg)	
		From the [3- ³ H]-5α-hydroxy sterol	From [26- ¹⁴ C]-cholest-7-en-3β-ol
1a	Crude homogenate	16.1	59.0
1b	Crude homogenate (boiled)	0	0
1c	Top half of 105000g supernatant	0	0
1d	Washed microsomal fraction	27.0	47.5

hydroxy sterol was made. Flasks containing [3α-³H]-cholest-7-ene-3β,5α-diol plus [26-¹⁴C]cholest-7-en-3β-ol were incubated in parallel for various times as shown in Fig. 1. The metabolism of the 5α-hydroxy sterol ceased after 30 min, whereas that of cholest-7-en-3β-ol continued for a further 30 min. Essentially

Fig. 1. Time-course for the conversion of cholest-7-en-3β-ol and cholest-7-ene-3β,5α-diol into cholesterol [26-¹⁴C]cholest-7-en-3β-ol (1.0 mg: 1.50 × 10⁵ d.p.m.) plus [3α-³H]cholest-7-ene-3β,5α-diol (1.0 mg: 1.39 × 10⁶ d.p.m.) in the same flask were aerobically incubated with rat liver homogenate (33.0 ml). At appropriate intervals portions (1.0 ml) were assayed for [3α-³H,26-¹⁴C]cholesterol by the dibromide method. O, Cholesterol biosynthesized from cholest-7-en-3β-ol; ●, cholesterol biosynthesized from cholest-7-ene-3β,5α-diol.

Table 3. Metabolism of cholest-7-en-3β-ol and cholest-7-ene-3β,5α-diol by preincubated rat liver homogenates

Samples of [3α-³H]cholest-7-en-3β,5α-diol (II) (7.79 × 10⁵ d.p.m./μmol and 6.18 × 10⁵ d.p.m./μmol for Expts. 1a and 1b respectively) plus [26-¹⁴C]cholest-7-en-3β-ol (I) (5.89 × 10⁴ d.p.m./μmol) were aerobically incubated for 180 min with rat liver homogenate (10.0 ml) that had been preincubated for various time-intervals. The [3α-³H,26-¹⁴C]cholesterol formed was isolated as the dibromide. In both the experiments the ³H/¹⁴C ratio of cholesterol obtained when the homogenate was not subjected to any preincubation has been normalized to the value of 1.0.

Expt.	Amount of substrates used (μg)		Time of preincubation (min)	Cholesterol formed (μg)		
	(II)	(I)		From 5α-[3α- ³ H]-hydroxy sterol	From [26- ¹⁴ C]-cholest-7-en-3β-ol	³ H/ ¹⁴ C ratio
1a	200	200	0	33.6	135.0	1.00
	200	200	20	25.8	127.0	0.81
	200	200	40	9.9	75.0	0.56
	200	200	60	5.4	50.0	0.43
	200	200	90	0.2	2.8	0.42
	200	200	0	0	0	—
			(boiled enzyme)			
1b	300	81	0	42.0	56.8	1.00
	300	81	20	34.8	55.2	0.89
	300	81	40	25.2	48.8	0.53
	300	81	60	10.8	31.2	0.47
	300	81	80	2.2	11.4	0.26
	300	81	0	0	0	—
			(boiled enzyme)			

the same result was obtained when this study was repeated with greater substrate concentrations.

In an allied experiment a comparative examination of the effect of partial denaturation of the rat liver homogenate on the conversion of the 5 α -hydroxy sterol and cholest-7-en-3 β -ol into cholesterol was made. The homogenate was aerobically preincubated at 37°C and after various time-intervals 10.0ml of the homogenate was transferred to successive flasks, each of which contained both the sterols to minimize differences in experimental conditions for the two substrates. As indicated in Table 3 the conversion of the 5 α -hydroxy sterol into cholesterol was more sensitive to the denaturation of the enzyme system than was the conversion of cholest-7-en-ol into cholesterol. This can be clearly seen by the significant decrease in the $^3\text{H}/^{14}\text{C}$ ratio of the biosynthesized cholesterol with increasing periods of preincubation. It is notable that in Expt. 1a (Table 3), as in previous experiments in Table 1, equal quantities of cholest-7-en-3 β -ol and the 5 α -hydroxy sterol being used as substrates, a smaller amount of cholesterol was biosynthesized from the 5 α -hydroxy sterol than from cholest-7-en-3 β -ol. When a similar experiment was performed, in which the relative amounts of cholest-7-en-3 β -ol and the 5 α -hydroxy sterol were adjusted to give approximately equal amounts of cholesterol, essentially the same results were obtained (Expt. 1b, Table 3).

Discussion

We have confirmed our previous finding that the 5 α -hydroxy sterol (II) is converted into cholesterol only under aerobic conditions and that this sterol (II) is a less efficient precursor of cholesterol than cholest-7-en-3 β -ol (I) (Dewhurst & Akhtar, 1967). Experiments carried out with doubly labelled cholest-7-en-3 β -ol have shown that the introduction of the 5,6-double bond in the biological conversion (I) $\rightarrow$ (IV) does not involve the loss of the 3 α -hydrogen atom, as might have been expected if the reaction involved first the hydroxylation of cholest-7-en-3 β -ol to the 5 α -hydroxy sterol (II) and then its dehydration by a pathway postulated for the corresponding reaction in the ergosterol series and outlined in Scheme 3 (Topham & Gaylor, 1972).

The most compelling evidence against the involvement of the 5 α -hydroxy sterol (II) as a direct intermediate in the conversions (I) $\rightarrow$ (IV) came from partial-denaturation experiments. It was shown that the enzyme system responsible for the conversion of the 5 α -hydroxy sterol (II) into cholesterol denatured faster than that for the corresponding conversion from cholest-7-en-3 β -ol (I). If the biological conversion of cholest-7-en-3 β -ol (I) into cholesterol occurred through the intermediary of

the 5 α -hydroxy sterol (II) then the factors adversely affecting the conversion of the 5 α -hydroxy sterol (II) into cholesterol (IV) must also similarly affect the formation of cholesterol from cholest-7-en-3 β -ol (I). This was not the case (Table 3). We therefore deduce that the 5-hydroxy sterol (II) is not involved in an intermediary role during the conversion of cholest-7-en-3 β -ol into cholesterol.

We may now examine the basis of the observation which has emphasized the role of the 5 α -hydroxy-ergostene derivative (VI) in the formation of the 5,6-double bond in ergosterol biosynthesis. Topham & Gaylor (1972) have described the purification of an enzyme system which converted 5 α -hydroxyergostene derivative (VI) into ergosterol under anaerobic conditions by a mechanism involving the loss of the 3 α -hydrogen atom. These workers argued that this loss of 3 α -hydrogen atom may explain the inability in our original work (Akhtar & Parvez, 1968) to detect any conversion of [3α - ^3H]ergosta-7,22-diene-3 β ,5 α -diol (VI) into ergosterol with anaerobically growing yeast cells. Topham & Gaylor (1972), however, overlooked the fact that in the same work a satisfactory conversion of [3α - ^3H]ergosta-7,22-diene-3 β ,5 α -diol into ergosterol was obtained by using aerobically growing yeast cells (Akhtar & Parvez, 1968).

More important, however, from a mechanistic viewpoint were the results obtained when [3α - ^3H]ergosta-7,22-dien-3 β -ol (V) was incubated with whole yeast cells. Up to 88% of the incubated radioactivity was recovered in the harvested cells and was associated with a sterol fraction; of this about 36% was actually incorporated into ergosterol (Akhtar & Parvez, 1968). If we make the extreme assumption that the 12% unaccounted-for radioactivity may represent the loss of 3 α - ^3H in the formation of ergosterol then a conservative conclusion may be drawn that for every 100 molecules of [3α - ^3H]ergosta-7,22-dien-3 β -ol (V) committed to ergosterol biosynthesis 75 completed their journey retaining the 3 α -hydrogen atom. If the enzyme system described by Topham & Gaylor (1972) for the metabolism of the 5 α -hydroxyergostene derivative (VI) was obligatorily involved in the biosynthesis *in vivo* then in the

Scheme 4.

conversion (V) $\rightarrow$ (VIII) the 3 α -hydrogen atom of (V) would have completely been lost. This was not the case.

In the light of these considerations we reaffirm our original conclusion that the introduction of the 5,6-double bond in cholesterol biosynthesis occurs through an O₂-dependent dehydrogenation process (Dewhurst & Akhtar, 1967). In view of close similarities between the rat liver and the yeast systems a similar mechanism may be involved for the corresponding reaction, (V) $\rightarrow$ (VIII), in ergosterol biosynthesis.

A hypothetical mechanism previously considered for the introduction of the 5,6-double bond in cholesterol biosynthesis is illustrated in Scheme 4. According to this, oxygen interacts with a highly reduced group —X: (e.g. a metal in its reduced form) of the enzyme to form the enzyme-oxygen species, which is then involved in the *cis* removal of the two hydrogen atoms as shown in Scheme 4.

It was suggested that this mechanism, predicting a *cis* elimination, may represent a general biological principle for the formation of ethylenic linkages involving C—H bonds not activated by electron-withdrawing groups (Dewhurst & Akhtar, 1967). This mechanism was consistent with observations on the biosynthesis of ethylenic linkages in oleic acid, linoleic acid, α -linolenic acid and arachidonic acid (Light *et al.*, 1962; Schroepfer & Bloch, 1965; Stoffel & Schiefer, 1966; Morris *et al.*, 1968). In particular Stoffel & Schiefer (1966) showed that in the biosynthesis of arachidonic acid O₂ could not be replaced by artificial electron acceptors. In the steroid field the *cis* elimination of hydrogen atoms in metabolism of cholesterol into cholest-7-en-3 β -ol was readily explicable by a process similar to Scheme 4 (Clayton & Edwards, 1963). Since the proposal of this mechanism, extensive studies, particularly at the University of Liverpool, have shown that the formation of *trans*-double bonds in the side chains of poriferasterol and ergosterol, as well as a variety of *cis*-double bonds in ring B of steroids by fungi, higher plants, algae, protozoa and mammals, all occur through the *cis* elimination of hydrogen atoms (Akhtar & Parvez, 1968; Smith *et al.*, 1968*a,b*; Bimpson *et al.*, 1969*a,b*; Goad *et al.*, 1969; Wilton & Akhtar, 1970; Zander & Caspi, 1970; Mulheirn *et al.*, 1971).

Scallen & Schuster (1968) and also Aberhart & Caspi (1971) considered an alternative mechanism, involving the transfer of one of the hydrogen atoms of the steroid (I) to nicotinamide nucleotides, for the desaturation reaction (I) $\rightarrow$ (III). Using 6 α -³H-labelled compound (I) Aberhart & Caspi (1971) showed that the 6 α -hydrogen atom of the substrate was not transferred to NADH; corresponding experiments with the 5 α -³H substrate were, however, not performed. The formation of the 7,8-double bond

in ecdysone biosynthesis also occurs with the overall *cis* removal of 7 β - and 8 β -hydrogen atoms. Preliminary evidence suggests that in this case the 7 β -hydrogen atoms may be transferred to nicotinamide nucleotides (Cook *et al.*, 1973).

We thank Dr. M. A. Parvez for the synthesis of some of the labelled intermediates used in this work and Professor K. A. Munday for his interest and encouragement.

References

- Aberhart, D. J. & Caspi, F. (1971) *J. Biol. Chem.* **246**, 1387–1392
- Akhtar, M. & Marsh, S. (1967) *Biochem. J.* **102**, 462–467
- Akhtar, M. & Parvez, M. A. (1968) *Biochem. J.* **108**, 527–531
- Akhtar, M., Watkinson, I. A., Rahimtula, A. D., Wilton, D. C. & Munday, K. A. (1969) *Eur. J. Biochem.* **9**, 107–111
- Bimpson, T., Goad, L. J. & Goodwin, T. W. (1969*a*) *Chem. Commun.* 297–298
- Bimpson, T., Goad, L. J. & Goodwin, T. W. (1969*b*) *Biochem. J.* **115**, 857–858
- Clayton, R. B. & Edwards, A. M. (1963) *J. Biol. Chem.* **238**, 1966–1972
- Cook, I. F., Lloyd-Jones, J. G., Rees, H. H. & Goodwin, T. W. (1973) *Biochem. J.* **136**, 135–145
- Dempsey, M. E. (1965) *J. Biol. Chem.* **240**, 4176–4188
- Dewhurst, S. M. & Akhtar, M. (1967) *Biochem. J.* **105**, 1187–1193
- Dewhurst, S. M., Kelly, R. S. A., Hudec, J. & Akhtar, M. (1967) *Biochem. J.* **105**, 1194
- Frantz, I. D. & Schroepfer, G. J., Jr. (1967) *Annu. Rev. Biochem.* **36**, 691–726
- Frantz, I. D., Davidson, A. G., Dullt, E. & Mobberley, M. L. (1959) *J. Biol. Chem.* **234**, 2290–2294
- Goad, L. J., Gibbons, G. F., Bolger, L. M., Rees, H. H. & Goodwin, T. W. (1969) *Biochem. J.* **114**, 885–892
- Light, R. J., Lennarz, W. J. & Bloch, K. (1962) *J. Biol. Chem.* **237**, 1793–1800
- Morris, L. J., Harris, R. V., Kelly, W. & James, A. T. (1968) *Biochem. J.* **109**, 673–678
- Mulheirn, L. J., Aberhart, D. J. & Caspi, E. (1971) *J. Biol. Chem.* **246**, 6556–6559
- Paliokas, A. M. & Schroepfer, G. J., Jr. (1968) *J. Biol. Chem.* **243**, 453–464
- Rees, H. H. & Goodwin, T. W. (1971) *Biosynthesis* **1**, 41–118
- Scallen, T. J. & Schuster, M. W. (1968) *Steroids* **12**, 683–687
- Schroepfer, G. J., Jr. & Bloch, K. (1965) *J. Biol. Chem.* **240**, 54–63
- Slaytor, M. & Bloch, K. (1965) *J. Biol. Chem.* **240**, 4598–4602
- Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1968*a*) *Chem. Commun.* 926–927
- Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1968*b*) *Chem. Commun.* 1259–1260
- Stoffel, W. & Schiefer, H.-G. (1966) *Hoppe-Seyler's Z. Physiol. Chem.* **245**, 41–51

- Topham, R. W. & Gaylor, J. L. (1972) *Biochem. Biophys. Res. Commun.* **47**, 180–186
- Wilton, D. C. & Akhtar, M. (1970) *Biochem. J.* **116**, 337–339
- Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968) *Biochem. J.* **106**, 803–810
- Zander, J. M. & Caspi, E. (1970) *J. Biol. Chem.* **245**, 1682–1687