The Stereochemistry of Hydrogen Elimination during 7,8-Double Bond Formation by *Tetrahymena pyriformis*

By D. C. WILTON AND M. AKHTAR

Department of Physiology and Biochemistry, University of Southampton, U.K.

(Received 17 September 1969)

It is shown that formation of the 7,8-double bond in the conversion of cholesterol into cholesta-5,7,22-trien-3 β -ol involves the removal of the 7 β - and 8 β -hydrogen atoms.

Dewhurst & Akhtar (1967) have discussed at least two types of fundamental mechanisms for rationalizing the formation of double bonds in biological systems. It was suggested that the mechanism for the introduction of C=C between two saturated carbon atoms, and involving non-activated C–H bonds, compulsorily requires the elimination of *cis*-orientated hydrogen atoms. In the present paper all C–H bonds that are not located α to an electron-withdrawing group (e.g. >C=O, –C=N– etc.) are referred to as non-activated bonds. In the biosynthesis of both ergosterol and cholesterol the insertion of the 5,6-double bond occurs with the elimination of the *cis*-orientated 5 α - and 6 α -hydrogen atoms. To test the general validity of our hypothesis we studied the stereochemical aspects of the insertion of the 7,8-double bond during the conversion of cholesterol (I) into cholesta-5,7,22-trien-3 β -ol (II) by whole cells of *Tetrahymena pyriformis*.

RESULTS AND DISCUSSION

Conner, Mallory, Landrey & Iyengar (1969) showed that whole cells of *Tetrahymena pyriformis* can efficiently convert cholesterol (I) into cholesta-5,7,22-trien-3 β -ol (II). The stereochemistry of the insertion of the 7,8-double bond in this conversion

was studied by using cholesterol stereospecifically labelled with ^3H at either the 7 α - or the 7 β -position. Wilton, Munday, Skinner & Akhtar (1968) have shown that incubation of 7-dehydrocholesterol with a 10000g supernatant of rat liver homogenate in the presence of tritiated water gave cholesterol containing radioactivity associated with the 8 β - and 7 α -hydrogen atoms in a 1:3 ratio. Tritiated cholesterol synthesized by this method was mixed with [26,27- ^{14}C]cholesterol to give a final $^3\text{H}/^{14}\text{C}$ ratio 1.00. The conversion of a sample of the cholesterol (I, $^3\text{H}/^{14}\text{C}$ ratio 1.00) gave cholesta-5,7,22-trien-3 β -ol (II, $^3\text{H}/^{14}\text{C}$ ratio 0.81) (Table 1). In this conversion, 20% of the original ^3H radioactivity was lost. The precursor cholesterol contained up to 25% of the ^3H radioactivity associated with the 8 β -hydrogen atom and the remaining 75% with the 7 α -hydrogen atom. In the conversion (I) $\rightarrow$ (II) the 8 β -hydrogen atom of the former must be removed. The retention of about 80% of the ^3H radioactivity in the conversion (I) $\rightarrow$ (II) suggests that, of the two 7-hydrogen atoms, it is the non-radioactivity-containing 7 β -hydrogen atom that is removed in the formation of the 7,8-double bond.

[7 β - ^3H]Cholesterol was synthesized by the method of Corey & Gregoriou (1959). When incubated as described above, this cholesterol (I,

Table 1. Conversion of [$7\alpha,8\beta\text{-}^3\text{H},26,27\text{-}^{14}\text{C}$]cholesterol and [$7\beta\text{-}^3\text{H},26,27\text{-}^{14}\text{C}$]cholesterol into cholesta-5,7,22-trien-3 β -ol by whole cells of *Tetrahymena pyriformis*

Substrate incubated	$^3\text{H}/^{14}\text{C}$ ratio of the precursor cholesterol	^{14}C specific radioactivity of the precursor cholesterol (c.p.m./ μmol)	$^3\text{H}/^{14}\text{C}$ ratio of cholesterol recovered from the incubation	$^3\text{H}/^{14}\text{C}$ ratio of cholesta-5,7,22-trien-3 β -ol acetate	^{14}C specific radioactivity of cholesta-5,7,22-trien-3 β -ol acetate (c.p.m./ μmol)	^3H lost from C-7 (%)
[$7\alpha,8\beta\text{-}^3\text{H}$]Cholesterol	1.00	562	1.01	0.81*	575	0
[$7\beta\text{-}^3\text{H}$]Cholesterol	1.00	715	1.02	0.10	705	90

* The cholesterol contained up to 25% of the ^3H in the 8β -position (Wilton *et al.* 1968). The compulsory elimination of the 8β -hydrogen during the conversion of cholesterol (I) into cholesta-5,7,22-trien-3 β -ol (II) results in the loss of this ^3H . Therefore the $7\alpha\text{-}^3\text{H}$ must have been undisturbed.

Scheme 1.

$^3\text{H}/^{14}\text{C}$ ratio 1.00) gave cholesta-5,7,22-trien-3 β -ol ($^3\text{H}/^{14}\text{C}$ ratio 0.10) that had lost 90% of the ^3H radioactivity. These results show that the insertion of the 7,8-double bond by the whole cells of *Tetrahymena pyriformis* occurs by a *cis*-elimination process involving the removal of the 7β - and 8β -hydrogen atoms.

A hypothetical mechanism that was proposed previously is shown in Scheme 1. The first stage in this mechanism is the formation of the enzyme-oxygen species (b), previously reported by Dewhurst & Akhtar (1967). It is the next stage of the reaction, involving a six-membered transition state, that imposes the stereochemical rigidity highlighted by Dewhurst & Akhtar (1967). Scheme 1 predicts that *cis*-hydrogen atoms may be removed not only in the formation of *cis*- but also of *trans*-double bonds. The *cis*-elimination of hydrogen atoms in the formation of the 22,23-*trans*-double bonds of certain steroids has been established by the extensive investigation of the Liverpool group (Smith, Goad & Goodwin, 1968; Bimpson, Goad & Goodwin, 1969). Other examples where the overall process occurs by a *cis*-elimination are the following: the insertion of the 5,6-double bond in ring B during the biosynthesis of cholesterol (Akhtar & Marsh, 1967; Dewhurst & Akhtar, 1967; Paliokas & Schroepfer, 1968) and ergosterol (Akhtar & Parvez, 1968); the biosynthesis of the

ethylenic linkage in oleic acid (Schroepfer & Bloch 1965) and arachidonic acid (Stoffel & Schiefer, 1966); the conversion of cholestanol into Δ^7 -cholestanol (Clayton & Edwards, 1963). Another type of mechanism for the insertion of double bonds seems to occur when substrates contain at least one C-H bond that is activated by the presence of an electron-withdrawing group located at the α -position. A brief description of this route was given by Dewhurst & Akhtar (1967).

EXPERIMENTAL

Materials. [$7\alpha,8\beta\text{-}^3\text{H}$]Cholesterol (^3H distribution: 75% 7α , 25% 8β) was prepared enzymically by the conversion of 7-dehydrocholesterol into cholesterol by using the 10000g supernatant of rat liver homogenate in the presence of tritiated water (Wilton *et al.* 1968). [$26,27\text{-}^{14}\text{C}$]Cholesterol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *Tetrahymena pyriformis* was kindly given by Dr Muriel Ord of the Department of Zoology, University of Southampton. All other materials were obtained as described by Wilton *et al.* (1968).

Incubation of cholesterol with *Tetrahymena pyriformis*. The method followed was as described by Conner *et al.* (1969). Cholesterol (40mg) in ethanol (8ml) was added to 4l of culture medium and, after inoculation, the cells were grown for 4 days with vigorous aeration. The cells were harvested, suspended in 500ml of water, and methanolic 10% (w/v) KOH (1000ml) was added. The mixture was refluxed for 2h under N_2 , then extracted

with 3×250 ml of light petroleum (b.p. 40–60°C)–diethyl ether (1:1, v/v). The combined extracts were washed with water, then dried and evaporated to dryness. The residue was spotted on a silica-gel plate (20 cm $\times$ 10 cm, 2 mm-thick layer), which was developed in acetone–light petroleum (b.p. 60–80°C) (1:4, v/v). The fluorescence-absorbing sterol band (R_f 0.4) was eluted with diethyl ether and the yield was determined spectrophotometrically ($\lambda_{\max}$ 281.5, ϵ 12100) (Conner *et al.* 1969). Conversions (I) $\rightarrow$ (II) of up to 35% were achieved by this incubation procedure.

After acetylation the material was spotted on a freshly prepared silica gel–10% AgNO_3 plate (20 cm $\times$ 10 cm, 1 mm-thick layer, fluorescent) and developed in benzene–light petroleum (b.p. 60–80°C) (3:2, v/v). The sterols were detected by spraying the edge of the plate with methanolic 10% phosphomolybdate. Both the cholesta-5,7,22-trien-3 β -ol band (R_f 0.2) and the cholesterol band (R_f 0.5) were eluted with diethyl ether. The ^{14}C specific radioactivity of compound (II) was determined from the u.v. spectrum ($\lambda_{\max}$ 281.5, ϵ 12700) (Conner *et al.* 1969).

Measurement of radioactivity (see Wilton, Rahimtula & Akhtar, 1969). All samples were counted in an Inter-technique SL40 liquid-scintillation counter. Corrected $^3\text{H}/^{14}\text{C}$ ratios were obtained by using the external standard and the appropriate computer programme.

REFERENCES

- Akhtar, M. & Marsh, S. (1967). *Biochem. J.* **102**, 462.
Akhtar, M. & Parvez, M. A. (1968). *Biochem. J.* **108**, 527.
Bimpson, T., Goad, L. J. & Goodwin, T. W. (1969). *Chem. Commun.* p. 297.
Clayton, R. B. & Edwards, A. M. (1963). *J. biol. Chem.* **238**, 1966.
Conner, R. L., Mallory, F. B., Landrey, J. R. & Iyengar, C. W. L. (1969). *J. biol. Chem.* **244**, 2325.
Corey, E. J. & Gregoriou, G. A. (1959). *J. Am. chem. Soc.* **81**, 3127.
Dewhurst, S. M. & Akhtar, M. (1967). *Biochem. J.* **105**, 1187.
Paliokas, A. M. & Schroepfer, G. J. (1968). *J. biol. Chem.* **243**, 453 (and references cited therein).
Schroepfer, G. J. & Bloch, K. (1965). *J. biol. Chem.* **240**, 54.
Smith, A. R. H., Goad, L. J. & Goodwin, T. W. (1968). *Chem. Commun.* p. 296.
Stoffel, W. & Schiefer, H. G. (1966). *Hoppe-Seyler's Z. physiol. Chem.* **345**, 41.
Wilton, D. C., Munday, K. A., Skinner, S. J. M. & Akhtar, M. (1968). *Biochem. J.* **106**, 803.
Wilton, D. C., Rahimtula, A. D. & Akhtar, M. (1969). *Biochem. J.* **114**, 71.