The Mechanism of C-4 Demethylation during Cholesterol Biosynthesis

EVIDENCE FOR A DECARBOXYLATION MECHANISM NOT INVOLVING A SCHIFF-BASE INTERMEDIATE

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The conversion of 4,4-dimethylcholest-7-enol into 4 α -methylcholest-7-enol by rat liver microsomal preparations involves the decarboxylation of a sterol 3-oxo-4 α -carboxylic acid. By using an ^{18}O -labelled substrate it was shown that this decarboxylation does not involve a Schiff-base intermediate.

It has long been established that the removal of the C-4 methyl groups that occurs during the biosynthesis of cholesterol from lanosterol is an oxidative process involving the decarboxylation of a sterol 3-oxo-4 α -carboxylic acid (Lindberg *et al.*, 1963; Swindell & Gaylor, 1968) as shown in Scheme 1. By analogy to previous known examples of the enzymic decarboxylation of such acids, two possible general mechanisms for this reaction may be proposed. In mechanism 1 (Scheme 2) the carbonyl must first condense with a suitable amine group on the enzyme surface to produce a protonated Schiff base as exemplified by the bacterial acetoacetate decarboxylase (Hamilton & Westheimer, 1959). The protonated Schiff base will provide an excellent electron sink to facilitate the decarboxylation process. Alternatively in mechanism 2 (Scheme 2) the participation of a bivalent metal ion potentiates the electron withdrawing capability of the carbonyl oxygen and may be used to stabilize an enolate intermediate during decarboxylation. This is the mechanism that has been proposed in the case of oxaloacetate decarboxylase from cod muscle (Kosicki & Westheimer, 1968).

The two mechanisms may be readily distinguished by virtue of the fact that mechanism 1 involves compulsory exchange of the oxygen atom at C-3 with the water of the medium during Schiff-base forma-

tion. No exchange of the oxygen will occur as a result of mechanism 2. The presence or absence of such an exchange may be demonstrated by carrying out the decarboxylation with ^{18}O -labelled substrates and analysing the product for the presence or absence of this heavy atom.

Experimental

The materials and methods were those described by Wilton *et al.* (1968)

Preparation of 4,4'-[3 β - ^{18}O]dimethylcholest-7-enol

4,4'-Dimethylcholest-7-enone was prepared from 4,4-dimethylcholesta-5,7-dienone by reduction with Raney nickel (Watkinson *et al.*, 1971) followed by oxidation with Jones reagent (Bowden *et al.*, 1946).

4,4'-Dimethylcholest-7-enone (5 mg) was dissolved in anhydrous tetrahydrofuran (0.1 ml) and to this was added H_2^{18}O (0.1 ml; 12.0% in excess) and ethylenediamine (10 μl). The mixture was heated in a sealed vial at 110°C for 24 h. Water was added and the sterol was extracted into diethyl ether, dried (over anhydrous Na_2SO_4) and evaporated to dryness. The residue was dissolved in anhydrous ether (20 ml), and excess of lithium aluminium hydride was added and the mixture was refluxed for 15 min. The sterol mixture was worked up in the usual way and the 4,4'-[3 β - ^{18}O]dimethylcholest-7-enol was purified

Scheme 1.

Scheme 2.

by t.l.c. (silica gel GF₂₅₄) by using light petroleum (b.p. 60–80°C)–acetone (17:3, v/v) as the developing solvent. The sterol was taken up in acetone and this solution was used for the incubations. The mass spectrum of the sterol gave an ¹⁸O content of 11.6% excess.

It should be noted that refluxing the sterol in the absence of ethylenediamine or in the presence of triethylamine (10 μl) gave a negligible incorporation of ¹⁸O. The triethylamine conditions when performed in ²H₂O gave over 50% of the dideuterated species.

Incubation procedure

Male Wistar albino rats (200g) were killed by cervical fracture and the excised livers were homogenized in 0.1M-sodium phosphate buffer, pH 7.4, containing 4mM-MgCl₂ in a loose-fitting piston-barrel-type homogenizer. The 105 000g microsomal fraction was prepared from this supernatant and resuspended in buffer at a concentration equivalent to 2g of liver/ml. Incubation conditions were as described in Table 1.

Isolation and purification of sterols

Incubations were terminated by the addition of

methanol (1 ml) and extracted twice with methylene chloride (3 ml). The combined extracts were dried (over Na₂SO₄), evaporated to dryness and the sterol band was purified on t.l.c. by developing in light petroleum (b.p. 60–80°C)–acetone (4:1, v/v) and removing the monomethyl and dimethyl sterol band (*R_F* 0.4–0.5), which was located running just ahead of the large cholesterol band.

Measurement of ¹⁸O content

The sterol mixture that had been partially purified by t.l.c. was subjected to combined g.l.c.–mass spectrometry on an AEI MS 30 instrument. Separation was on a 274cm (9ft) column of 3% OV1 operating at 330°C; this system allowed analysis of both the re-isolated dimethyl and monomethyl sterols.

All measurements were also carried out on samples derived from incubations of ¹⁶O-labelled 4,4-dimethylcholest-7-enol to allow the calculation of ¹⁸O content of the ¹⁸O samples.

Results and Discussion

The substrate of choice for this work was 4,4'-dimethylcholest-7-enol (I), which is demethylated by

Table 1. Incubation of 4,4'-[3 β - 18 O]dimethylcholest-7-enol with rat liver microsomal preparations

Each incubation (2ml) contained 50 μ g of sterol solubilized in 2.5mg of Tween 80, 5 μ mol of GSH, 5 μ mol of glucose 6-phosphate, 1.25 μ mol of NAD $^{+}$ and NADPH, 2×10^{-8} katal of glucose 6-phosphate dehydrogenase, 200 μ mol of phosphate buffer, pH 7.4, 30 μ mol of nicotinamide, 4 μ mol of MgCl $_2$ and a microsomal preparation equivalent to 1g of liver. Incubations were for 1h in air at 38°C. The re-isolated dimethyl sterol (I) and the biosynthesized 4 α -methyl sterol (III) were separated by g.l.c. and analysed by mass spectrometry.

Experiment	18 O content of 4,4-dimethylcholest-7-enol (I) (%)	18 O content of 4 α -methylcholest-7-enol (III) (%)	Retention of 18 O in compound (III) (%)
1	11.3	8.9	79
2	11.0	8.4	76

rat liver microsomal preparations as shown in Scheme 1 to give the corresponding 4 α -methylcholest-7-enol (III) and thence to give cholest-7-enol. Incubation with an excess of substrate leads to an accumulation of this monomethylated sterol, which may be separated from the starting material and endogenous cholesterol by g.l.c. and analysed by mass spectrometry.

The labelled substrate (11.6% atom excess of 18 O) was obtained by exchanging the oxygen function of 4,4'-dimethylcholest-7-enone with H $_2$ 18 O and then reducing the ketone to 4,4'-dimethylcholest-7-enol. This labelled substrate was incubated with rat liver microsomal fractions under aerobic conditions in the presence of NAD $^{+}$ and NADPH and the resulting sterols were extracted, purified by t.l.c. and analysed by g.l.c.-mass spectrometry. Analysis of the substrate and the monomethyl product revealed that there was only a small loss of 18 O label during the course of the demethylation sequence (Table 1). This small loss of 18 O was not surprising in view of the fact that the reaction sequence (I) to (III) involves the intermediacy of the carbonyl compounds (II) and (IV), in which the oxygen is potentially

labile. The loss by exchange of carbonyl oxygens has been observed in other systems (Heron & Caprioli, 1974; D. C. Wilton, unpublished work).

The fact that the majority of the 18 O has been retained rules out the possibility of Schiff-base formation (mechanism 1) and is therefore consistent with a mechanism involving the direct involvement of the electron withdrawing capacity of the carbonyl oxygen (mechanism 2). This electron movement may be facilitated by either enzyme-mediated protonation of the carbonyl oxygen to give an enol intermediate or by stabilization of an enolate intermediate by using a bivalent metal ion. In mechanism 2 we have indicated the latter possibility by taking the analogy with cod muscle oxaloacetate decarboxylase. However, this does not preclude a mechanism independent of a metal ion and indeed both this sterol enzyme system (Rahimtula & Gaylor, 1972) and other oxaloacetate decarboxylases (Dean & Bartley, 1973) have, as yet, no apparent requirement for bivalent metal ions.

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