The Incorporation of a Hydrogen Atom at C-15 of Cholesterol Biosynthesized from Squalene

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Cholesterol is biosynthesized from squalene in the presence of tritiated water. Chemical degradation reveals that a considerable percentage of the total radioactivity is present at C-15. This result confirms the previous observations on the involvement of a C-15 hydrogen atom in cholesterol biosynthesis.

To examine the mechanism of C-H bond formation in enzymic reactions we studied the biosynthesis of cholesterol from squalene in tritiated water (Akhtar, Wilton & Munday, 1966; Akhtar, Watkinson, Rahimtula, Wilton & Munday, 1969b). The information available at the time suggested that in the conversion (I) $\rightarrow$ (II) new C-H bonds may be formed at positions 3, 4, 7, 8, 9, 14, 24 and 25. Accordingly cholesterol was biosynthesized from squalene in the presence of tritiated water and, as expected, contained a considerable amount of radioactivity. The degradation of this cholesterol revealed that after removal of the radioactivity from positions 3, 4, 7, 8, 9, 14, 24 and 25 about 40% of the radioactivity remained unaccounted for. This observation suggested that some hitherto unknown reaction(s) might be involved in cholesterol biosynthesis. We predicted that the remaining radioactivity must be located at C-15. The reasons for this prediction were first discussed by Wilton (1967), and the main features of the argument that resulted in the proposal of Scheme 1 for the involvement of one of the C-15 hydrogen atoms in cholesterol biosynthesis have been reported by Akhtar *et al.* (1969b). In accordance with the requirement of Scheme 1 it has been shown that in the biosynthesis of cholesterol from [2*RS*- $^3\text{H}_2$]mevalonic acid one of the C-15 hydrogen atoms is removed (Akhtar *et al.*

1969b; Canonica *et al.* 1968; Gibbons, Goad & Goodwin, 1968). Also, the formation of a diene of the type (V) under the conditions of cholesterol biosynthesis (Watkinson & Akhtar, 1969) and the conversion of the diene into cholesterol have been established (Akhtar *et al.* 1969b; Canonica *et al.* 1968). This paper shows that in accordance with our previous predictions position 15 is labilized during the biosynthesis of cholesterol from squalene (Wilton, 1967; Akhtar *et al.* 1969b).

RESULTS

Cholesterol was biosynthesized from squalene in the presence of tritiated water by using the 10000g supernatant of rat liver homogenate. Cholesterol obtained in this manner should contain at least two types of radioactive hydrogen atoms: those derived from a proton source and those derived from the 4B-position of NADPH (Wilton, Munday, Skinner & Akhtar, 1968). This cholesterol, after purification, was diluted to a total of 25g. with non-radioactive cholesterol and subjected to three series of degradations.

Degradation A: determination of radioactivity at positions 7, 8, 9, 14 and 15 of cholesterol. Cholesterol (II) (16g.; 28300 c.p.m./m-mole) was acetylated and oxidized by chromium trioxide to give 3 β -hydroxycholesta-5-en-7-one acetate (VII) (4g.;

(I)

(II)

Scheme 1.

25890 c.p.m./m-mole), showing that 8.5% of the radioactivity was located at C-7. The latter compound (VII) (3.8 g.; 25890 c.p.m./m-mole) on treatment with lithium aluminium hydride followed by benzylation with benzoyl chloride and pyridine gave cholesta-5-ene-3 β ,7 β -diol dibenzoate, which on pyrolysis in dimethylaniline gave cholesta-5,7-dien-3 β -ol benzoate (VIII) (2 g.; 25045 c.p.m./m-mole). Position 8 thus contained 3.0% of the total radioactivity.

A part of the cholesta-5,7-dien-3 β -ol benzoate (VIII) (800 mg.; 25045 c.p.m./m-mole) was hydrogenated in the presence of Raney nickel catalyst and the resulting compound was hydrolysed with lithium aluminium hydride and acetylated to give cholesta-7-en-3 β -ol acetate (IX) (450 mg.; 25025 c.p.m./m-mole). The latter compound (IX) (300 mg.; 25025 c.p.m./m-mole) was converted into 8,9-epoxy-3 β -hydroxycholestan-7-one acetate (X) (15 mg.; 22360 c.p.m./m-mole) and 8,14-epoxy-3 β -hydroxycholestan-7-one acetate (XI) (9 mg.; 23350 c.p.m./m-mole), thus demonstrating that 9.5% and 6.1% of the total radioactivity was present at C-9 and C-14 respectively.

Another part of the cholesta-5,7-dien-3 β -ol benzoate (VIII) (1.0 g.; 25045 c.p.m./m-mole) was

isomerized with hydrogen chloride gas to cholesta-7,14-dien-3 β -ol benzoate (600 mg.). The latter compound on epoxidation with *m*-chloroperbenzoic acid followed by treatment with hydrochloric acid gave 3 β -hydroxycholesta-8(14)-en-15-one benzoate (XII) (150 mg.; 11460 c.p.m./m-mole). The conversion (VIII) $\rightarrow$ (XII), in addition to removing the C-15 hydrogen atoms, could in principle result in the labilization of the C-16 and C-9 hydrogen atoms. It has been shown that in the biosynthesis of cholesterol from mevalonic acid the C-16 hydrogen atoms are not involved (Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969). Thus the loss of 42% of the radioactivity encountered in the conversion (VIII) $\rightarrow$ (XII) should therefore represent either the amount of radioactivity located at C-15 or the combined radioactivity at C-15 and C-9. Since C-9 contains only 9.5% of the total radioactivity we deduced that 32.5–42% of the radioactivity is located at C-15. This conclusion was fully substantiated by an alternative degradation.

A portion of the cholesta-5,7-dien-3 β -ol benzoate (VIII) (100 mg.; 24045 c.p.m./m-mole) was converted into cholesta-8,14-dien-3 β -ol benzoate (XIII) (70 mg.; 12600 c.p.m./m-mole). The loss of radioactivity associated with this conversion (44.0%)

represents radioactivity present at C-9 (9.5%), C-14 (6.0%) and C-15 (28.5%). The removal of the radioactivity from C-15 in the above transformation is due to a protonation-deprotonation mechanism as shown by the conversion (XIV)→(XVI). The

results are in complete agreement with our previous predictions and show that the C-15 position is involved in cholesterol biosynthesis (Wilton, 1967; Akhtar *et al.* 1969b).

Degradation B: determination of radioactivity at

positions 3 and 4 of cholesterol. Cholesterol (II) (1.0g.; 28300 c.p.m./m-mole) was oxidized with Jones' reagent (Bowden, Heilbron, Jones & Weedon, 1946) to cholest-5-en-3-one (550mg.). The latter compound on methylation with methyl iodide and potassium *tert.*-butoxide followed by reduction with lithium aluminium hydride gave 4,4-dimethyl-cholesterol (XVII; 250mg.; 25330 c.p.m./m-mole). This degradation demonstrates that 10.5% of the total radioactivity was present at C-2, C-3 and C-4.

Degradation C: determination of radioactivity at positions 24 and 25 of cholesterol. Cholesterol (II) (7.5g.; 19195 c.p.m./m-mole) was converted into dehydroepiandrosterone acetate (XVIII) (108mg.; 16650 c.p.m./m-mole) by the method of Hershberg (1948) with a loss of 13.2% of the radioactivity of the parent cholesterol. This radioactivity (13.2%) must have been located in the side chain of cholesterol and was incorporated during the saturation of the $\Delta^{24,25}$ -double bond. The dehydroepiandrosterone acetate was further diluted with an equal amount of non-radioactive material and then subjected to basic equilibration. Treatment for 1 hr. with methanolic 5% potassium hydroxide at 37°, a procedure known to labilize the hydrogen atoms at C-16 (Fishman, 1966), resulted in a complete retention of radioactivity, establishing that there was no radioactivity present at C-16. The non-involvement of hydrogen atoms at C-16 during cholesterol biosynthesis has already been established by using [5*RS*-³H₂]mevalonic acid (Akhtar *et al.* 1969a).

DISCUSSION

The results described above show that in the biosynthesis of cholesterol from squalene in the presence of tritiated water radioactivity is incorporated at C-15. The degradative methods used in the present work do not allow a precise estimation of the radioactivity located at C-15 to be made. These methods, however, satisfactorily show that this position must contain 32–42% of the total radioactivity, thus demonstrating the involvement of C-15 in cholesterol biosynthesis and providing further support for the mechanism of Scheme 1.

From the cumulative degradative work, which involved the determination of radioactivity at 22 of the 27 carbon atoms of cholesterol (Table 1), we could account for the location of up to 90% of the incorporated radioactivity. The remaining 10% of the radioactivity could be due to the experimental error. However, since this would require that the error in all cases is in the same direction, this seems unlikely.

The recent demonstration that 7,9-dienes of the type (XIX) are converted into cholesterol raises the possibility that the C-11 position contains the missing radioactivity (Rahimtula, Wilton & Akhtar,

1969). One may argue that in addition to the well-known pathway leading to the formation of lanosterol from squalene there exists another route as shown in Scheme 2. This involves the cyclization of squalene to give the cation (XXII) and this after the removal of a C-11 hydrogen atom gives the Δ^9 -steroid parkeol (XXIII) (see also Rees, Goad & Goodwin, 1968). The insertion of a double bond between C-7 and C-8 by a mechanism analogous to that already described for the formation of the Δ^5 -double bond of cholesterol (Akhtar & Marsh, 1967; Dewhurst & Akhtar, 1967) would then give the 7,9-diene (XIX). The work of Rahimtula *et al.* (1969) would require that, in the conversion of the 7,9-diene system (XIX) into cholesterol in tritiated water, radioactivity is incorporated at C-11 as a result of C–H bond formation. Another possible cyclization product of squalene would be cycloartenol (Rees *et al.* 1968), which on cleavage of the cyclopropane ring could incorporate radioactivity from the medium into the C-19 methyl group.

EXPERIMENTAL

Materials were obtained, enzymic systems (10000g supernatant of rat liver homogenate) were prepared, incubations were carried out and processed, and cholesterol was isolated and purified as described by Wilton *et al.* (1968). Squalene was obtained from Kodak Ltd., Kirkby, Liverpool, and was purified by twice processing it through the thiourea adduct. The product was stored in the dark at –15°.

Incubation procedure. Samples (5ml.) of a 10000g supernatant of rat liver homogenate were incubated under oxygen at 37° for 4 hr. in the presence of 0.02 ml. of squalene, glucose 6-phosphate (15 μ moles), GSH (15 μ moles), NAD⁺ (8 μ moles) and NADP⁺ (7 μ moles). A total of 4 c of tritiated water was used to give labelled cholesterol, which, after purification, contained 2000000 c.p.m. This cholesterol was subsequently diluted with a total of 25g. of non-radioactive cholesterol. In blank experiments, which were performed in the absence of squalene, the radioactivity incorporated

Table 1. *Distribution of radioactivity in biosynthetic cholesterol*

Position in cholesterol	Percentage of total radioactivity
C-2 + C-3 + C-4	10.5
C-7	8.5
C-8	3.0
C-9	9.5
C-14	6.0
C-15	32.5–42.0
C-20 to C-27	13.2
	83.2–92.7

Scheme 2.

into cholesterol represented 11% of that when squalene was present.

Measurement of radioactivity. All samples were counted in an Intertechnique SL40 liquid-scintillation counter at about 50% counting efficiency. Quenching was corrected by external standardization. The scintillation fluid consisted of 0.8% 2-(4-*tert.*-butylphenyl)-5-(4-biphenyl)-1-oxo-3,4-diazole in toluene.

Conversion of cholesterol into dehydroepiandrosterone acetate. Cholesterol (7.5g.) was converted into dehydroepiandrosterone acetate semicarbazone (230mg.) by the method of Hershberg (1948). The semicarbazone (230mg.) was dissolved in acetic acid (0.66ml.) at 110°. Anhydrous sodium acetate (70mg.) in acetic acid (0.33ml.) and pyruvic acid-water (1:1, w/w) (154mg.) were added. After 10 min. at 110°, 0.3 ml. of water was added followed by a further 0.6 ml., which was added dropwise up to 25 min. Finally water (1.0 ml.) containing a seed of dehydroepiandrosterone acetate was added and the solution was

cooled. After crystallization had started acetic acid-water (1:3, v/v) (2ml.) was added and the mixture was cooled and filtered. Recrystallization from acetone-light petroleum (b.p. 60–80°) gave dehydroepiandrosterone acetate (108mg.).

Conversion of cholesterol into 3 β -hydroxycholest-8(14)-en-15-one benzoate. The methods for this series of degradations have been described by Akhtar *et al.* (1969b).

Conversion of cholesterol into 4,4-dimethylcholesterol. The preparation of dimethylcholesterol has been described by Akhtar *et al.* (1969b).

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