A Novel Olefinic Rearrangement

THE ENZYMIC CONVERSION OF CHOLESTA-7,9-DIEN-3 β -OL INTO CHOLESTA-8,14-DIEN-3 β -OL

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1. [3 α -³H]cholesta-7,9-dien-3 β -ol is converted in high yield into cholesterol by a 10000g_{av.} supernatant fraction of rat liver homogenate.
2. Incubation of cholesta-7,9-dien-3 β -ol with [4-³H]NADPH and rat liver microsomal fractions under anaerobic conditions resulted in ³H being incorporated into the 14 α -position of cholest-7-en-3 β -ol.
3. Under anaerobic conditions in the absence of NADPH cholesta-7,9-dien-3 β -ol was isomerized into cholesta-8,14-dien-3 β -ol by rat liver microsomal fractions.

Our interest in the mechanisms of biosynthesis in the steroid field led us to investigate the possible role of a 7,9-diene system of the type (I) in cholesterol biosynthesis. The diene system of the type (I) is present in the naturally occurring mammalian steroids agnosterol (IV) and dihydroagnosterol (III) (Fieser & Fieser, 1959), and both compounds (III) and (IV) have been biosynthesized from [¹⁴C]acetate (Clayton & Bloch, 1956). We have reported that cholesta-7,9-dien-3 β -ol (I) is efficiently converted into cholesterol (V) by a 10000g_{av.} supernatant of rat liver homogenate (Rahimtula *et al.*, 1969). The precise pathway for the conversion of compound (I) into compound (V) had not been determined, but it was assumed that the 7,9-diene would first be saturated by an NADPH-linked reductase to give cholest-7-en-3 β -ol (II); the conversion of the latter compound into cholesterol may then be accomplished by the participation of the normal pathway. The results reported below show that the metabolism of compound (I) into compound (V) occurs through a more complex pathway involving the intermediacy of an 8,14-diene system (VI).

Results and Discussion

Incubation of cholesta-7,9-dien-3 β -ol (I) with [4-³H]NADPH in the presence of rat liver microsomal fractions

As a result of extensive investigations from this laboratory on the saturation of double bonds in biological systems (Watkinson *et al.*, 1971a), we would predict that, if the metabolic conversion of compound (I) into compound (II) occurs by the direct reduction of the $\Delta^{9(11)}$ -double bond, then initial protonation at C-11 would occur followed by the transfer of a hydrogen atom from the cofactor to the 9 α -position.

Cholesta-7,9-dien-3 β -ol (I) (800 μ g) was incubated

anaerobically with a washed microsomal fraction of rat liver homogenate in the presence of [4-³H]-NADPH. The resulting biosynthetic cholest-7-en-3 β -ol (II; 34220 d.p.m./mmol) was oxidized to the 11-ketone (VII; 38080 d.p.m./mmol). In this conversion all the original hydrogen atoms located at C-9 and C-11 of the starting material (I) were removed without loss of radioactivity. The degradation of cholest-7-en-3 β -ol into the 8,9-epoxide (VIII; 40620 d.p.m./mmol) was also accompanied by the complete retention of radioactivity. However, the conversion of compound (II) into the 8,14-epoxide (IX; 210 d.p.m./mmol) resulted in the complete loss of the label. This cumulative evidence shows that the NADPH-derived hydrogen atom was present at the 14 α -position of the sterol nucleus. This unexpected result can be explained by assuming that the cholesta-7,9-dien-3 β -ol (I) first rearranges into cholesta-8,14-dien-3 β -ol (VI) or cholesta-7,14-dien-3 β -ol (X); enzymic reduction of the $\Delta^{14,15}$ -double bond in the presence of [4-³H]NADPH would result in the incorporation of radioactivity at the C-14 position. This conjecture was subjected to direct experimental examination.

Incubation of [3 α -³H]cholesta-7,9-dien-3 β -ol (I) with the 10000g_{av.} supernatant and microsomal fractions of rat liver homogenate

We have previously demonstrated that when [3 α -³H]cholesta-7,9-dien-3 β -ol is incubated with a 10000g_{av.} supernatant fraction of rat liver under aerobic conditions there is a 56% conversion of radioactivity into cholesterol, whereas under anaerobic conditions cholest-7-en-3 β -ol (II) is produced in at least 20% yield (Rahimtula *et al.*, 1969). To ascertain whether in fact the primary product of the metabolism of compound (I) was a diene containing

- (I) $R = H$
 (II) 9,11-Dihydro-compound (I)
 (III) $R = CH_3$
 (IV) 24,25-Dehydro-compound (III)

a $\Delta^{14,15}$ -double bond, compound (I) was incubated with a 10000 g_{av} supernatant enzyme system in the presence or absence of a 'trap' of non-radioactive cholesta-8,14-dien-3 β -ol (VI; 2mg). The percentage

conversion into the 'trap' and into cholesterol was determined for these incubations. As shown in Table 1, in the absence of the 'trap', 70% of the recovered radioactivity could be accounted for in the cholesterol

Table 1. Formation of $[3\alpha\text{-}^3\text{H}]\text{cholesta-8,14-dien-3}\beta\text{-ol}$ (VI) from $[3\alpha\text{-}^3\text{H}]\text{cholesta-7,9-dien-3}\beta\text{-ol}$ (I)

$[3\alpha\text{-}^3\text{H}]\text{cholesta-7,9-dien-3}\beta\text{-ol}$ ($100\mu\text{g}$; 700000d.p.m.) was incubated with either 9 ml of a $10000g_{av}$. supernatant or 5 ml of a microsomal preparation of rat liver homogenate. 'Trapping' experiments were carried out with the $10000g_{av}$. supernatant preparation by using non-radioactive cholesta-8,14-dien-3 β -ol (2 mg).

Incubation conditions	Conversion into cholesta-8,14-dien-3 β -ol (%)	Conversion into cholesterol (%)
$10000g_{av}$. supernatant system	8.1	69.9
$10000g_{av}$. supernatant system + 'trap'	48.1	2.6
$10000g_{av}$. supernatant system (boiled) + 'trap'	5.4	1.0
Microsomal fractions	75.8	—
Microsomal fractions (boiled)	9.3	—
Buffer without enzyme system	2.3	—

band, whereas 8% was found associated with the 8,14-diene band. However, in the presence of the 'trap', conversion into cholesterol was only 2.6%, whereas 48% of the radioactivity was recovered in the 8,14-diene band. Incubation of compound (I) with rat liver microsomal fractions in the absence of NADPH gave a 76% conversion into compound (VI), thus establishing that the enzyme system responsible for the isomerization of compound (I) is located in the microsomal fraction of the liver. The chromatographic procedure used in the above experiments does not allow the separation of cholesta-8,14-dien-3 β -ol (VI) and cholesta-7,14-dien-3 β -ol (X). To distinguish between these two possible products, radioactive diene isolated from the above experiment was acetylated and mixed with non-radioactive cholesta-8,14-dien-3 β -ol acetate, then was recrystallized from methanol-diethyl ether, during which process there was almost complete retention of radioactivity. On the other hand, recrystallization of authentic tritiated cholesta-7,14-dien-3 β -ol acetate with the 8,14-diene acetate resulted in a loss of 40% of the radioactivity after four recrystallizations. Thus there is an enzyme system present in the rat liver microsomal fractions which isomerizes cholesta-7,9-dien-3 β -ol into cholesta-8,14-dien-3 β -ol. The precise mechanism for this isomerization has yet to be determined, but a number of possibilities exist. Thus, for example initial protonation at C-7 followed by suitable rearrangements would result in the formation of the more stable cholesta-8,14-dien-3 β -ol as shown in Scheme 1.

In view of the above observations on the metabolism of cholesta-7,9-dien-3 β -ol, it is interesting to speculate on how dihydroagnosterol (IV) is converted into cholesterol, since a rearrangement in this compound cannot occur until 14 α -demethylation has taken place. One possibility is that the $\Delta^{9(11)}$ -double bond of dihydroagnosterol is first reduced to give Δ^7 -dihydrolanosterol and this is converted into cholesterol in the normal fashion. To check this point

dihydroagnosterol was incubated with a $10000g_{av}$. supernatant fraction of rat liver homogenate under anaerobic conditions. After termination of the incubation, the sterols were extracted in the usual way and separated by t.l.c. on silica-gel GF 254 plates, impregnated with 10% (w/w) AgNO_3 . There was no conversion of dihydroagnosterol (IV) into Δ^7 -dihydrolanosterol (XI). Therefore it seems probable that 14-demethylation occurs with the 7,9-diene system still intact to give a triene (XII). We have previously shown that the enzyme system catalysing the reduction of the $\Delta^{14,15}$ -double bond of sterols has a broad specificity and can function with substrates having a second olefinic linkage in either the $\Delta^{7,8}$ - or $\Delta^{8,9}$ -positions (Watkinson *et al.*, 1971*b*). It is therefore conceivable that the enzyme can also reduce the 14,15-double bond in the triene (XII) to give compound (I), which after rearrangement is metabolized to cholesterol in the normal way.

Experimental

Materials were obtained and radioactivity was measured as described previously (Bloxham *et al.*, 1971). $[3\alpha\text{-}^3\text{H}]\text{cholesta-7,9-dien-3}\beta\text{-ol}$ was prepared and the acetylation and chromatographic analysis of the sterol acetates were undertaken by the methods of Rahimtula *et al.* (1969). Cholesterol was isolated as its dibromide derivative (Wilton *et al.*, 1968), or located on the t.l.c. plate by spraying with phosphomolybdic acid in methanol.

Preparative methods

Preparation of homogenates and incubation procedure. Male Wistar albino rats (150–200 g) were killed by cervical fracture. The excised livers were homogenized in cold 0.1 M-potassium phosphate buffer, pH 7.3, containing 30 mM-nicotinamide and 4 mM-MgCl₂ (2 ml/g of liver). A manually operated

Scheme 1. Possible pathway for the conversion of cholesta-7,9-dien-3β-ol into cholesta-8,14-dien-3β-ol

piston-barrel homogenizer (clearance, 1 mm) was used. A 10000 g_{av} cell-free supernatant was prepared by centrifuging the homogenate at 10000 g_{av} for 15 min. The supernatant was decanted and used in all 10000 g_{av} supernatant incubations. The 105000 g_{av} microsomal fractions were prepared by centrifuging the 10000 g_{av} supernatant prepared as described above at 105000 g_{av} for 1 h. The pellet obtained was suspended in the 0.1M-potassium phosphate buffer described above (1 ml/g of liver). The preparation was kept at 0–5°C throughout (Wilton *et al.*, 1968).

Incubations were performed in 25 ml Erlenmeyer flasks at 37°C with gentle shaking. Steroids were dissolved in Tween 80 (25 mg for the 5 ml microsomal incubation and 50 mg for the 9 ml 10000 g_{av} supernatant incubation). All incubations were terminated by the addition of an equal volume of methanolic 10% (w/v) KOH. The steroids were extracted, after the addition of non-radioactive carrier steroid, with 3 × 25 ml of light petroleum (b.p. 40–60°C)–diethyl ether (1:1, v/v). The combined extracts were washed

with water three times, dried and evaporated to dryness. The steroids were then analysed chromatographically and, in the case of cholesterol, also isolated as the dibromide.

Preparation of cholesta-7,9-dien-3β-ol and cholesta-7,9-dien-3β-ol acetate. These were prepared by the method of Heusser *et al.* (1952). Cholest-7-en-3β-ol acetate (230 mg) in purified chloroform (3 ml) was added to a solution of mercuric acetate (450 mg) in acetic acid (6 ml) and the mixture was stirred in the dark at room temperature for 5 h. The precipitated mercurous acetate was filtered and the filtrate was diluted with water and extracted with ether. The extract was washed successively with water, saturated NaHCO₃ solution and saturated NaCl solution, dried and evaporated to dryness. The residue was spotted on two silica-gel plates (HF 254) impregnated with 20% (w/w) AgNO₃ (20 cm × 10 cm; 1 mm thick) and was run three times in light petroleum (b.p. 40–60°C)–benzene (1:1, v/v). The lower band with R_F 0.7, located under u.v. light, was extracted with

ether and the ether extract was evaporated to dryness. The residue was crystallized three times from ether-methanol to give pure cholesta-7,9-dien-3 β -ol acetate (70mg). The acetate, dissolved in ether (8ml), was refluxed for 15 min with lithium aluminium hydride (30mg) to give, after crystallization from methanol, cholesta-7,9-dien-3 β -ol.

Preparation of 3 β -acetoxycholestane-8 α ,9 α -epoxy-7-one. This was prepared by the method of Fieser (1953). The cholest-7-en-3 β -ol obtained from the experiment described in the Results and Discussion section was acetylated overnight in a pyridine-acetic anhydride mixture. The cholest-7-en-3 β -ol acetate obtained was diluted with non-radioactive carrier. A solution of cholest-7-en-3 β -ol acetate (300mg) in acetic acid (15ml) was cooled to 30°C and treated with a solution of CrO₃ (450mg) in 65% (v/v) acetic acid (1ml). The temperature was controlled to about 25°C by cooling the flask in tap water. After being left overnight, the solution was diluted with water (6ml), which caused the separation of the 8,9-epoxide. The material was collected, washed with water and dried (yield 25mg) and then was crystallized twice from methanol (yield 15mg).

Preparation of 3 β -acetoxycholestane-8 α ,14 α -epoxy-7-one. Ether extraction of the diluted acetic acid mother liquor of the above oxidation (3 β -acetoxycholestane-8 α ,9 α -epoxy-7-one) gave a yellow gum. This was spotted on a silica-gel (HF 254) plate (20cm $\times$ 10cm; 1mm thick) and run once in light petroleum (b.p. 40–60°C)-acetone (9:1, v/v). After development with iodine, the band corresponding to R_F 0.35 was extracted with ether and the ether extract evaporated to dryness. The residue was crystallized twice from light petroleum (b.p. 30–40°C) (yield 25mg).

Preparation of 3 β -acetoxycholest-8-en-11-one. The cholest-7-en-3 β -ol acetate obtained above was converted into cholesta-7,9-dien-3 β -ol acetate by the method also outlined above. A solution of cholesta-7,9-dien-3 β -ol acetate (250mg) in ether (10ml) was mixed with a solution of *m*-chloroperbenzoic acid

(130mg) in ether (2ml) and left at room temperature in the dark for 24h. The mixture was washed with cold 2M-NaOH and saturated NaCl solution, dried and evaporated to dryness. The residue was dissolved in dry benzene (6ml) and treated with 2 drops of BF₃-ether complex (Fieser & Fieser, 1960). After 1 min saturated NaHCO₃ solution was added, the mixture was shaken and the benzene layer evaporated to dryness *in vacuo*. The resulting oil was refluxed in acetic acid (8ml) for 3h. Excess of water was added and the mixture was extracted with ether. The extract was washed successively with water, saturated NaHCO₃ solution and saturated NaCl solution, dried and evaporated to dryness. The residue was spotted on a silica-gel HF254 plate (20cm $\times$ 10cm; 1mm thick) and run twice in benzene. The band with R_F 0.55, corresponding to the 11-ketone, was eluted with ether and the extract was evaporated to dryness. The residue was crystallized twice from methanol to give pure 3 β -acetoxycholest-8-en-11-one (yield 100mg).

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