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The Formation of Cholest-5-ene-3β,26-diol as an Intermediate in the Conversion of Cholesterol into Bile Acids by Liver Mitochondria

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1. When [^{14}C]cholesterol was incubated with rat liver mitochondria, radioactive 26-hydroxycholesterol, 3β-hydroxychol-5-enoic acid and other bile acids were isolated from the incubation mixture. 2. In the absence of added 26-hydroxycholesterol, the specific radioactivity of the 26-hydroxycholesterol formed from [^{14}C]cholesterol during the incubation was higher than that of the 3β-hydroxychol-5-enoic acid. Addition of increasing amounts of 26-hydroxycholesterol led to a progressive fall in the specific radioactivity, and to a progressive increase in the mass, of the 3β-hydroxychol-5-enoic acid recovered at the end of the incubation. 3. It is concluded that 26-hydroxycholesterol is an intermediate in the formation of 3β-hydroxychol-5-enoic acid from cholesterol. 4. Comparison of the specific radioactivity of the 26-hydroxycholesterol formed in the incubation mixture with that of the added [^{14}C]cholesterol indicates that endogenous cholesterol in mitochondria is accessible to cholesterol 26-hydroxylase.

Mitropoulos & Myant (1967*a*) have shown that [4-^{14}C]cholesterol incubated with rat liver mitochondria gives rise to radioactive bile acids, including 3β-hydroxychol-5-enoic acid and lithocholic acid, and a radioactive steroid with the R_F value of cholest-5-ene-3β,26-diol (26-hydroxycholesterol) on t.l.c. This finding suggested that cholesterol can be converted into lithocholic acid in rat liver by a pathway which includes hydroxylation at C-26 to give 26-hydroxycholesterol, oxidative cleavage of the side chain of 26-hydroxycholesterol to give 3β-hydroxychol-5-enoic acid and the conversion of this acid into lithocholic acid by saturation of the Δ^5 double bond and epimerization of the hydroxyl group at C-3. In support of this suggestion, 3β-hydroxychol-5-enoic acid was shown to be converted into lithocholic acid when injected intravenously into rats, or when incubated with rat-liver mitochondria (Mitropoulos & Myant, 1967*b*). In the present paper we describe experiments designed to test the hypothesis that 26-hydroxycholesterol is an intermediate in the formation of 3β-hydroxychol-5-enoic acid from cholesterol in suspensions of rat liver mitochondria.

Methods and Materials

Preparation of rat liver fractions

The rats used were males of the Wistar strain, weighing 150–175g, and were fed on M.R.C. Diet no. 18 (Bruce & Parkes, 1946). They were killed at about 10:00 a.m. on the day of the experiment by breaking their necks. For the preparation of the mitochondrial suspension, the livers were removed immediately, chilled in ice and perfused with ice-cold 0.25M-sucrose to remove blood from the tissue. They were then minced in a stainless-steel mincer and homogenized in 4vol. of ice-cold 0.25M-sucrose. The homogenate was centrifuged at 800*g* for 10min and the supernatant was centrifuged at 9000*g* for 15min to give a supernatant fraction (S_9 fraction) and a mitochondrial pellet. The mitochondrial pellet was suspended in 2vol. of ice-cold 0.25M-sucrose and centrifuged at 9000*g* for 15min to give the washed mitochondrial pellet. The pellet was suspended in ice-cold 0.25M-sucrose to give the washed mitochondrial suspension, 1ml containing the mitochondria from 2g of fresh liver. The S_9 fraction was centrifuged at 104000*g* for 60min to give a supernatant (S_{104} fraction) and a microsomal pellet. The microsomal suspension was prepared from livers homogenized in 0.1M-potassium phosphate buffer (pH7.4) with and without 1mM-EDTA, as described by Mitropoulos & Balasubramaniam (1972).

Conditions of incubation

The standard incubation mixture (pH8.5) contained: tris–HCl buffer (120mM), ATP (2.7mM), NAD^+ (0.5mM), GSH (3.2mM), $MgCl_2$ (2.6mM), potassium citrate (4.6mM), 0.5ml of washed mitochondrial suspension (containing 8–10mg of protein and 350–450nmol of total cholesterol), 0.5ml of a 1% solution of bovine serum albumin and 0.5ml of

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S_{104} fraction. The final volume was 3.5ml. [4-^{14}C]-Cholesterol (20nmol with 1μCi) was added to the incubation mixture as a solution in 0.03ml of acetone. 26-Hydroxycholesterol was added at the beginning of the incubation in 0.5ml of 1% bovine serum albumin. The incubations were carried out in paired flasks at 37°C, with air as the gas phase and with constant shaking. Control flasks were prepared by boiling the mitochondrial suspension and the S_{104} fraction for 10min before addition to the incubation mixture, and were incubated in parallel with the unboiled flasks. The incubation mixture used for the experiments with microsomal preparations contained, in a final volume of 5ml, 0.1M-potassium phosphate buffer (pH7.4), nicotinamide (30mM), $MgCl_2$ (5mM), NADP (1mM), glucose 6-phosphate (10mM), 0.5ml of microsomal suspension and sufficient glucose 6-phosphate dehydrogenase to generate 1μmol of NADPH/min. The [4-^{14}C]cholesterol (20nmol with 1μCi) was added as a solution in acetone. The production of $^{14}CO_2$ from [26-^{14}C]cholesterol in suspensions of mitochondria was measured in Warburg flasks by the method of Mitropoulos & Myant (1965*a*).

Determination of the activities of marker enzymes in the mitochondrial and microsomal suspensions showed that 13% of the microsomal glucose 6-phosphatase and 4% of the microsomal NADPH–cytochrome *c* reductase was present in the mitochondrial preparation, and that 1.2% of the mitochondrial succinate–cytochrome *c* reductase was present in the microsomal preparation.

Analysis of the incubation mixture (*see Scheme* 1)

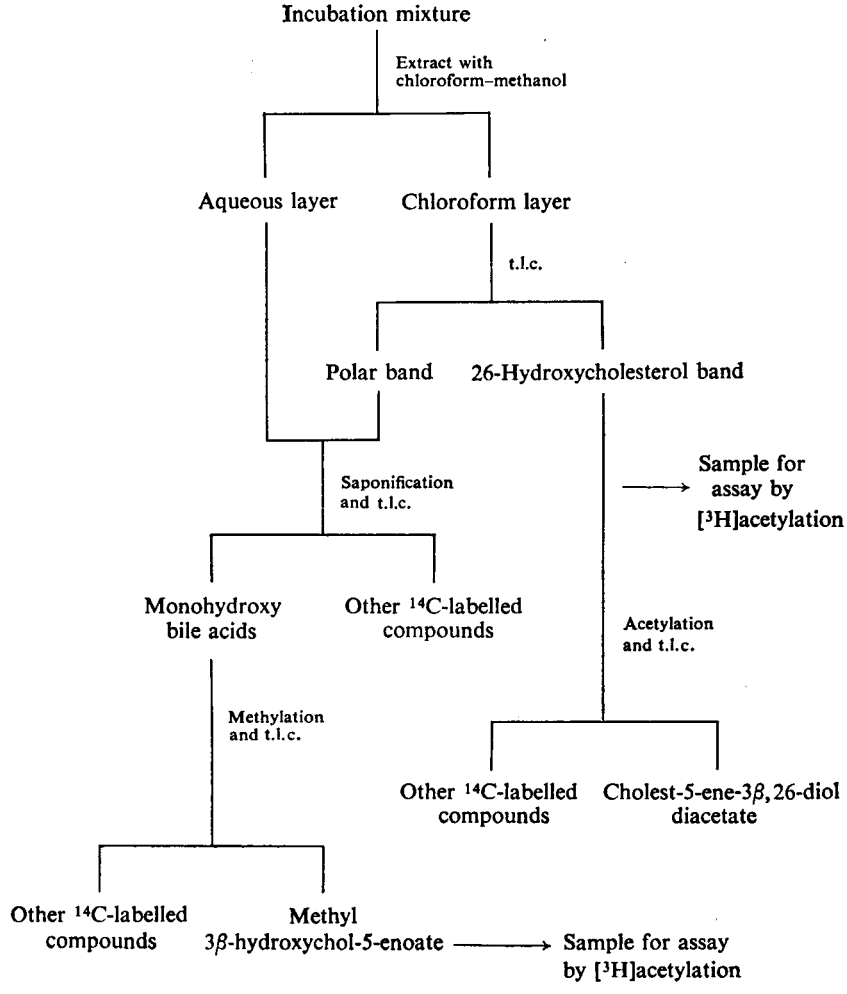
For the analysis of the standard incubation mixture, the incubation was terminated by adding 0.5ml of 0.5M-H_2SO_4 and 20vol. of chloroform–methanol (2:1, v/v). The mixture was shaken vigorously and the protein precipitate removed by filtration through a sintered-glass filter. The filtrate was washed with 0.2vol. of 0.9% NaCl and allowed to separate into an aq. methanol and a chloroform layer. The radioactive compounds in the chloroform layer were separated by t.l.c. with diethyl ether as solvent and with a marker of 26-hydroxycholesterol run in a separate lane. The chromatography was carried out at 5°C without prior equilibration of the solvent. The radioactive bands were located by radioautography and the position of the marker was found by spraying the marker lane with the ammonium molybdate–perchloric acid spray of Wagner *et al.* (1961) after covering the remainder of the plate with aluminium foil. The radioactive bands were scraped from the plate and eluted through a sintered-glass filter with 50ml of chloroform–methanol (2:1, v/v). The incorporation of ^{14}C into 26-hydroxycholesterol and 3β-hydroxychol-5-enoic acid was determined as follows.

Radioactivity was assayed in a sample of the eluate from the radioactive band corresponding to the 26-hydroxycholesterol marker. A known fraction of the remainder was evaporated to dryness and acetylated. The acetylated steroids were separated by t.l.c., with a marker of cholest-5-ene-3β,26-diol diacetate run in a separate lane. The radioactive bands were located by radioautography. The band corresponding to the marker of cholest-5-ene-3β,26-diol diacetate and the remainder of the lane were then removed and eluted separately, and the amount of radioactivity was determined in each of the two eluates. The proportion of the total radioactivity present as cholest-5-ene-3β,26-diol diacetate was determined, and this value was used to calculate the radioactivity due to 26-hydroxycholesterol in the chloroform extract of the incubation mixture. Authentic samples of 26-hydroxycholesterol and 25-hydroxycholesterol acetylated under the same conditions gave single bands of cholest-5-ene-3β,26-diol diacetate and 3β-acetoxycholest-5-en-25-ol respectively when submitted to t.l.c. No bands corresponding to the unacetylated sterols were observed.

The eluate from the polar band at the origin obtained on t.l.c. of the chloroform extract of the incubation mixture was combined with the aqueous phase and the mixture was evaporated to dryness. The residue was dissolved in 6ml of 1M-NaOH and saponified in an autoclave at 120°C for 3h. The mixture was acidified and extracted with diethyl ether, and the ether extract was submitted to t.l.c. on Kieselgel H with solvent no. 1 of Hofmann (1962). The radioactive band containing the monohydroxy bile acids was eluted and a sample of the eluate was taken for radioassay. The remainder of the eluate was methylated and submitted to t.l.c. on Kieselgel H impregnated with 5% $AgNO_3$, with chloroform–acetone (24:1, v/v) as solvent and with markers of methyl lithocholate and methyl 3β-hydroxychol-5-enoate. The radioactive bands were eluted and the proportions of the total radioactivity due to the two methyl esters were determined. These values were used to calculate the radioactivity due to lithocholic acid and 3β-hydroxychol-5-enoic acid in the incubation mixture.

Determination of the mass of 26*-hydroxycholesterol and* 3β*-hydroxychol-5-enoic acid*

A sample of the eluate from the 26-hydroxycholesterol band obtained on t.l.c. of the choloroform extract of the incubation mixtures and a sample of the eluate containing methyl 3β-hydroxychol-5-enoate were evaporated and acetylated with [^{3}H]-acetic anhydride (Mitropoulos & Balasubramaniam, 1972). The [^{14}C]cholest-5-ene-3β,26-diol [^{3}H]diacetate formed was purified by t.l.c. on Kieselgel H with chloroform at 5°C and the radioactive diacetate



Scheme 1. *Steps in the isolation and assay of* 26-*hydroxycholesterol and* 3β-*hydroxychol*-5-*enoic acid*

located by radioautography, eluted and rechromatographed with benzene–ethyl acetate (10:1, v/v). The $^3H/^{14}C$ specific-radioactivity ratio was determined in the diacetate obtained after rechromatography.

The methyl 3β-[^{3}H]acetoxy[^{14}C]chol-5-enoate obtained by acetylation of methyl 3β-hydroxychol-5-enoate was purified by t.l.c. on Kieselgel H with benzene–ethyl acetate (10:1, v/v) and the radioactive acetoxy compound located by radioautography, eluted and rechromatographed with chloroform at 5°C. The $^3H/^{14}C$ specific-radioactivity ratio was determined in the monoacetate obtained after rechromatography.

The amount (μmol) of 26-hydroxycholesterol in the incubation mixture (T) was calculated from the equation:

$$T = \left(\frac{AR - B}{S}\right) \quad (1)$$

where A = the amount (d.p.m.) of 26-hydroxy[^{14}C]-cholesterol in the incubation mixture; R = the $^3H/^{14}C$ specific-radioactivity ratio in the purified diacetate of 26-hydroxycholesterol; S = the specific radioactivity of the [^{3}H]acetic anhydride (d.p.m./μmol); B = the blank value for ^{3}H (d.p.m.), determined as described below.

Each experiment included a pair of flasks containing the standard incubation mixture with no mitochondria and no S_{104} fraction. The contents of the flasks were extracted and submitted to the complete procedure for assay of the mass of 26-hydroxycholesterol. ^3H recovered in the final eluate after acetylation with [^3H]acetic anhydride was measured and the value expressed in d.p.m. derived from the whole volume of incubation mixture.

The amount (μmol) of 3β-hydroxychol-5-enoic acid present in the incubation mixture (T) was calculated from the equation:

$$T = 2\left(\frac{AR-B}{S}\right) \qquad (2)$$

where A = the amount (d.p.m.) of 3β-hydroxy[^{14}C]-chol-5-enoic acid in the incubation mixture; R = the $^3H/^{14}C$ specific-radioactivity ratio in the purified methyl 3β-acetoxychol-5-enoate; S = the specific radioactivity of the [^3H]acetic anhydride (d.p.m./μmol); B = the blank value for ^3H (d.p.m.), determined in the same way as for eqn. (1), except that the blank incubation mixture was submitted to the complete procedure for assay of the mass of 3β-hydroxychol-5-enoic acid.

When the method was tested by assaying known amounts of 26-hydroxycholesterol and methyl 3β-hydroxychol-5-enoate by acetylation with [^3H]acetic anhydride, the method was found to be accurate to within 9.6±2.4% of the amount of 3β-hydroxychol-5-enoic acid added and to within 16±6% of the amount of 26-hydroxycholesterol added.

Analytical methods

Cholesterol was determined by the method of Clark *et al.* (1968), protein by the biuret method, NADPH–cytochrome *c* reductase by the method of Phillips & Langdon (1962), succinate cytochrome *c* reductase by the method of Green *et al.* (1955) and glucose 6-phosphatase by the method of de Duve *et al.* (1955). Radioactivity was measured with a Beckman LS-250 liquid-scintillation spectrometer. Cholest-5-ene-3β,26-diol diacetate and methyl 3β-acetoxychol-5-enoate were assayed by g.l.c. with a Varian model 2700 gas chromatograph with a flame-ionization detector and with cholesterol as an internal standard.

For assay of cholest-5-ene-3β,26-diol diacetate the stationary phase was 1.5% QF-1, supported on Varaport 80–100 mesh, column temperature was 230°C, detector temperature was 272°C, injector temperature was 255°C and the carrier gas was N_2 at a flow rate of 38ml/min. Under these conditions detector response was proportional to mass of cholesterol and of cholest-5-ene-3β,26-diol diacetate.

For assay of methyl 3β-acetoxychol-5-enoate the stationary phase was 1.5% SE-30, supported on Varaport 80–100 mesh, column temperature was 220°C, detector temperature was 275°C, injector temperature was 275°C and the carrier gas was N_2 at a flow rate of 50ml/min. Under these conditions detector response was proportional to mass of cholesterol and of methyl 3β-acetoxychol-5-enoate.

Materials

ATP, NAD^+, NADP and GSH were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Kryptogenin was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Lithocholic acid and 3β-hydroxychol-5-enoic acid were obtained from Steraloids Ltd., Croydon, Surrey, U.K. The monoacetate of 25-hydroxycholesterol was a generous gift from Dr. P. L. Perlman of the Schering Corp., Bloomfield, N.J., U.S.A. [4-^{14}C]Cholesterol (50μCi/μmol), [26-^{14}C]cholesterol (55μCi/μmol) and [^3H]acetic anhydride (100μCi/μmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The [^{14}C]cholesterol was purified by t.l.c. at 5°C on Kieselgel H with diethyl ether as solvent, followed by rechromatography with benzene–ethyl acetate (3:7, v/v). The [^3H]acetic anhydride was diluted with non-radioactive acetic anhydride, purified and assayed for specific radioactivity by the method of Mitropoulos & Balasubramaniam (1972).

26-Hydroxycholesterol was prepared from kryptogenin by the method of Scheer *et al.* (1956); immediately before addition to the incubation mixture, 3mg of the crystalline product was purified by t.l.c. with diethyl ether at 5°C, eluted and suspended in bovine serum albumin solution. Cholest-5-ene-3β,26-diol diacetate (m.p. 130–131°C) was prepared by acetylation of 26-hydroxycholesterol.

Methyl 3β-hydroxychol-5-enoate (m.p. 145.5–146.5°C) and methyl lithocholate were prepared by methylating the free acids with diazomethane. Methyl 3β-acetoxychol-5-enoate (m.p. 159–160°C) was prepared by acetylating the methyl ester of the bile acid with acetic anhydride.

Results

Radioactive metabolites formed from [^{14}C]cholesterol in mitochondrial suspensions

Formation of 26-hydroxycholesterol. Standard incubation mixtures containing [4-^{14}C]cholesterol were incubated for 2h without added 26-hydroxycholesterol. They were then extracted with chloroform–methanol and the chloroform layer was submitted to t.l.c. with ether. Radioautographs of the chromatograms showed five main bands of blackening: a polar band at the origin containing free and conjugated bile acids, a band with R_F 0.21, a band with R_F 0.47 corresponding to a marker of 26-hydroxycholesterol, a band with R_F 0.77 due to

cholesterol and a band with R_F 0.88 due to cholesteryl esters. The band with R_F 0.47 contained 1.5–4% of the total radioactivity extracted from the incubation mixture. This band was eluted and a portion of the eluate was evaporated to dryness, acetylated and submitted to t.l.c. with chloroform. Radioautographs of the chromatograms showed three bands of blackening: a band with R_F 0.15 corresponding to a marker of 3β-acetoxycholest-5-en-25-ol, a band with R_F 0.25 and a band with R_F 0.45 corresponding to cholest-5-ene-3β,26-diol diacetate. The band with R_F 0.45 contained 50–60% of the total radioactivity eluted from the whole chromatogram. When the mitochondria and S_{104} fraction were boiled before incubation, radioautographs made from thin-layer chromatograms of the acetylated radioactive products showed a faint band of blackening with R_F 0.40, but no blackening in the zone corresponding to the marker of cholest-5-ene-3β,26-diol diacetate.

In Expt. 1 (Table 1), portions of the eluates from the band with R_F 0.47 obtained by t.l.c. of the chloroform layer of the extracts from all the unboiled incubation mixtures were combined. 26-Hydroxycholesterol (21 mg) was dissolved in the pooled eluate and the eluate was evaporated to dryness. The residue was recrystallized to constant specific radioactivity from ethyl acetate. After three recrystallizations the specific radioactivity of the crystals fell to 55% of that of the residue before crystallization and remained constant thereafter. When the boiled incubation mixtures from Expt. 1 were treated in the same way, the specific radioactivity of the crystals fell progressively throughout five recrystallizations.

Effect of 26-hydroxycholesterol on the accumulation of radioactive products. Three experiments were carried out to determine the effect of increasing concentrations of non-radioactive 26-hydroxycholesterol on the accumulation of 26-hydroxy[^{14}C]cholesterol and of 3β-hydroxy[^{14}C]chol-5-enoic acid in standard incubation mixtures incubated for 2h with [4-^{14}C]-cholesterol.

In the first experiment, radioactivity was measured in the aqueous layer of the initial extract of the incubation mixture, and in the 26-hydroxycholesterol and bile acids isolated from the chloroform layer. In this experiment, the radioactive compounds present in the aqueous layer of the initial extract were not included in the analysis of bile acids. T.l.c. of the saponified bile acid fraction gave three radioactive bands due to mono-, di- and tri-hydroxy acids. When the monohydroxy acids were eluted and methylated, t.l.c. gave two radioactive bands due to methyl 3β-hydroxychol-5-enoate and methyl lithocholate, as described previously (Mitropoulos & Myant, 1967*a*).

The amounts of radioactivity present in the aqueous layer, in 26-hydroxycholesterol and in the bile acid fractions, are shown in Table 1. In the absence of added 26-hydroxycholesterol, 1.1% of the total ^{14}C was recovered as 26-hydroxycholesterol, 3.8% was recovered as monohydroxy acids and 2.4% was recovered as trihydroxy and dihydroxy acids. In the fraction containing the monohydroxy acids most of the radioactivity was due to 3β-hydroxychol-5-enoic acid. When 26-hydroxycholesterol was added to the incubation mixture at increasing concentrations,

Table 1. *Expt. 1: effect of 26-hydroxycholesterol on accumulation of radioactive products formed from [4-^{14}C]-cholesterol incubated with rat liver mitochondria*

26-Hydroxycholesterol was purified by t.l.c. after acetylation. The bile acids were obtained as the polar band on t.l.c. of the chloroform layer of the extract of the incubation mixture. The monohydroxy acids were purified by t.l.c. of the methyl esters. For other experimental details see the Methods and Materials section. All values are means of duplicate estimations and are expressed as percentages of the total radioactivity recovered in the combined aqueous and chloroform layers of the extract of the incubation mixture. Each flask contained approx. 1.78×10^6 d.p.m.

26-Hydroxycholesterol added (μg)	Radioactivity recovered from incubation mixture					
	Aqueous layer	Chloroform layer				
		26-Hydroxycholesterol	3β-Hydroxycholenoic acid	Lithocholic acid	Dihydroxy acids	Trihydroxy acids
None	9.10	1.10	2.57	1.23	1.08	1.35
25	7.32	2.35	2.13	1.10	1.53	1.85
75	5.42	3.82	2.10	0.91	1.15	1.47
150	4.08	5.00	0.80	0.61	1.17	1.16
None (boiled)	0.21	0.22*	0.01	0.02	0.22	0.12

* Radioactivity eluted from the zone extending from R_F 0.40 to R_F 0.45 after t.l.c. of acetylated products (see the Results section).

there was a progressive decrease in radioactivity recovered in the aqueous layer and a progressive increase in that recovered as 26-hydroxycholesterol. There was also a decrease in the percentage of the total radioactivity recovered in the monohydroxy acids, but no consistent change in that recovered in the dihydroxy and trihydroxy acids.

The incubation mixture in the boiled flasks was treated in the same way as the unboiled preparations, except that the band eluted from the chromatogram of the acetylated radioactive products extended from R_F 0.40 to R_F 0.45. Therefore the 26-hydroxycholesterol fraction shown for the boiled preparations in Table 1 included the acetylated radioactive compound with R_F 0.40. This fraction contained 0.2% of the total radioactivity in the chloroform–methanol extract of the incubation mixture.

In Expts. 2 and 3, in addition to the measurements of radioactivity, the mass of 26-hydroxycholesterol and of 3β-hydroxychol-5-enoic acid in the incubation mixture was determined at the end of the incubation. In these experiments, the analysis of 3β-hydroxychol-5-enoic acid was carried out after combining the aqueous layer of the initial extract with the eluate from the polar band obtained after t.l.c. of the chloroform layer (see Scheme 1). After chromatography of the extract from the boiled incubation mixture, the radioactive band with the R_F of 26-hydroxycholesterol was eluted, acetylated and rechromatographed. As in Expt. 1, the zone eluted from the t.l.c. plate obtained after rechromatography extended from R_F 0.40 to R_F 0.45. Each experiment included a pair of flasks in which the incubation mixture was extracted with chloroform at zero time (i.e. without incubation).

The results are shown in Table 2. In both experiments, 26-hydroxycholesterol added to the incubation mixture at increasing concentrations led to a progressive increase in the amount of radioactivity recovered as 26-hydroxycholesterol, but had no consistent effect on that recovered as 3β-hydroxychol-5-enoic acid. In the flasks with no added 26-hydroxycholesterol about 2nmol of 26-hydroxycholesterol was recovered at the end of the incubation, but none was detectable in the boiled incubation mixture after incubation in parallel with the unboiled preparations, or in the incubation mixtures extracted at zero time.

In the flasks containing no added 26-hydroxycholesterol, 7–10nmol of 3β-hydroxychol-5-enoic acid was recovered after incubation. When 26-hydroxycholesterol was added at increasing concentrations, there was a progressive increase in the mass of 3β-hydroxychol-5-enoic acid recovered.

Table 2. *Expts. 2 and 3: effect of 26-hydroxycholesterol on the specific radioactivities of 26-hydroxycholesterol and 3β-hydroxychol-5-enoic acid recovered after incubation of [4-^{14}C]cholesterol with rat liver mitochondria*

26-Hydroxycholesterol was purified by t.l.c. after acetylation. 3β-Hydroxychol-5-enoic acid was purified by t.l.c. of the methyl ester. For other experimental details see the Methods and Materials section. All values are means of duplicate estimations. Each flask contained approx. 2.52×10^6 d.p.m. n.a., Not assayed.

Expt. no.	26-Hydroxycholesterol added (μg)*	26-Hydroxycholesterol recovered from the incubation mixture			3β-Hydroxychol-5-enoic acid recovered from the incubation mixture		
		(% of added ^{14}C)	(nmol)	(d.p.m./nmol)	(% of added ^{14}C)	(nmol)	(d.p.m./nmol)
2	None	0.68	2.1	8180	1.38	7.2	4820
	25	0.90	54.2	417	1.95	18.4	2669
	75	1.07	125	214	2.25	27.6	2049
	150	1.45	253	155	1.94	30.2	1739
	None (boiled)	0.55†	0	—	0.26	n.a.	—
3	None	0.60	2.3	6488	2.00	10.0	5050
	25	0.62	19.9	789	1.54	6.9	5596
	75	1.01	55.5	458	1.63	10.5	3921
	150	1.46	97.8	404	1.52	20.1	2058
	None (boiled)	0.29†	0	—	0.19	n.a.	—

* Owing to the method of preparation of the stock suspension of 26-hydroxycholesterol, the absolute amounts of 26-hydroxycholesterol added to the flasks in Expts. 2 and 3 may have been less than the values shown, but the relative amounts were as shown in the table.

† Radioactivity from the zone extending from R_F 0.40 to R_F 0.45 after t.l.c. of acetylated products in each experiment (see the Results section).

The specific radioactivity of 26-hydroxycholesterol was higher than that of 3β-hydroxychol-5-enoic acid in the flasks with no added 26-hydroxycholesterol. When 26-hydroxycholesterol was added at increasing concentrations, there was a progressive fall in specific radioactivity of both compounds. However, the fall was less marked in 3β-hydroxychol-5-enoic acid than in 26-hydroxycholesterol, so that in all flasks with added 26-hydroxycholesterol the specific radioactivity of 3β-hydroxychol-5-enoic acid was greater than that of 26-hydroxycholesterol.

Further evidence for the identity of the acetylated radioactive compounds prepared for determination of mass. In Expt. 2, samples of the [^{14}C]cholest-5-ene-3β,26-diol [^{3}H]diacetate prepared from each flask for the final step in the determination of the mass of 26-hydroxycholesterol (see Scheme 1) were combined and dissolved in ethyl acetate. Portions of this sample subjected to g.l.c. with 1.5% SE-30 or 1.5% QF-1 (see the Methods and Materials section) showed in every case a peak with the same retention time as authentic cholest-5-ene-3β,26-diol diacetate. Another portion of the sample was mixed with 14.3mg of cholest-5-ene-3β,26-diol diacetate and the ^{3}H/^{14}C specific-radioactivity ratio was then determined in the crystals obtained by repeated recrystallization from chloroform–methanol. The ratio did not change significantly during four recrystallizations (Table 3).

Samples of the methyl 3β-[^{3}H]acetoxy[^{14}C]chol-5-enoate prepared from each flask of Expt. 2 and purified by t.l.c. were pooled and dissolved in ethyl acetate. Portions of this sample subjected to g.l.c. with 1.5% SE-30 or 1.5% QF-1 showed in every case a peak with the same retention time as authentic methyl 3β-acetoxychol-5-enoate. Another portion of the sample was mixed with 20.8mg of methyl-3β-acetoxychol-5-enoate and recrystallized repeatedly from chloroform–methanol. After the first crystallization the ^{3}H/^{14}C specific-radioactivity ratio fell to 66% of the ratio determined in the original mixture, but remained constant on further recrystallization (Table 4). The fall in specific-radioactivity ratio was due mainly to a fall in ^{3}H radioactivity caused by the elimination of ^{3}H contaminants during the first

Table 3. *Specific radioactivity of [^{14}C]cholest-5-ene-3β,26-diol [^{3}H]diacetate recrystallized with authentic diacetate*

Samples of the [^{14}C]cholest-5-ene-3β,26-diol [^{3}H]diacetate prepared from each flask in Expt. 2 were pooled and mixed with cholest-5-ene-3β,26-diol diacetate before recrystallization. The dried crystals were dissolved in chloroform–methanol and samples were taken for radioassay and for assay of mass by g.l.c. For experimental details see the Methods and Materials section.

		Specific radioactivity (d.p.m./mg)		
No. of crystallizations	Wt. of crystals (mg)	^{14}C	^{3}H	^{3}H/^{14}C ratio
Original mixture	14.3	735	25074	34.1
1	11.7	678	25448	37.5
2	9.1	638	24227	38.0
3	6.4	623	24941	40.0
4	3.6	672	26973	40.0

Table 4. *Specific radioactivity of methyl 3β-[^{3}H]acetoxy[^{14}C]chol-5-enoate recrystallized with authentic methyl ester*

Samples of the methyl 3β-[^{3}H]acetoxy[^{14}C]chol-5-enoate prepared from each flask in Expt. 2 were pooled and mixed with methyl 3β-acetoxychol-5-enoate before recrystallization. The dried crystals were dissolved in chloroform–methanol and samples were taken for radioassay and for assay of mass by g.l.c. For experimental details see the Methods and Materials section.

		Specific radioactivity (d.p.m./mg)		
No. of crystallizations	Wt. of crystals (mg)	^{14}C	^{3}H	^{3}H/^{14}C ratio
Original mixture	20.8	563	1264	2.25
1	17.0	541	806	1.49
2	13.7	497	707	1.42
3	10.3	464	661	1.43
4	5.4	519	762	1.47

Table 5. *Effect of 26-hydroxycholesterol on the production of $^{14}CO_2$ from [26-^{14}C]cholesterol incubated with rat liver mitochondria*

Results are given as mean values from paired incubations. For experimental details see the Methods and Materials section.

26-Hydroxycholesterol added (μg)	Radioactivity recovered as $^{14}CO_2$ (% of total)
None	0.66
200	0.32
400	0.16
800	0.06
None (boiled)*	0

* Incubation mixture with boiled mitochondria and boiled S_{104} fraction.

crystallization. In the determination of the mass of 3β-hydroxychol-5-enoate in the incubation mixture, the value obtained from the blank sample was used to correct for these contaminants (see the Methods and Materials section).

Effect of 26-hydroxycholesterol on the production of $^{14}CO_2$ from [26-^{14}C]cholesterol. When [26-^{14}C]-cholesterol was incubated with the mitochondrial suspension for 6h in Warburg flasks, about 0.7% of the total radioactivity in the incubation mixture was recovered as $^{14}CO_2$ (Table 5). When 26-hydroxycholesterol was added in increasing amounts at the beginning of the incubation, radioactivity recovered as CO_2 decreased. No $^{14}CO_2$ was recovered during incubation of [26-^{14}C]cholesterol in incubation mixtures containing boiled mitochondria and boiled S_{104} fraction.

Incubation of [4-^{14}C]cholesterol in microsomal suspensions

To test the ability of liver microsomes to form 26-hydroxycholesterol from cholesterol, [4-^{14}C]-cholesterol was incubated with rat-liver microsomes under the conditions described in the Methods and Materials section. At the end of the incubation, the incubation mixture was extracted with chloroform–methanol and the chloroform phase of the extract submitted to t.l.c. on Kieselgel H with diethyl ether at 5°C, and with a marker of 26-hydroxycholesterol run in an adjacent lane. When the microsomes were prepared from livers homogenized in the presence of EDTA, no significant radioactivity was found in the eluate from the zone on t.l.c. corresponding to the marker of 26-hydroxycholesterol. When the homogenization was carried out in the absence of EDTA, significant radioactivity was present in the zone corresponding to 26-hydroxycholesterol on t.l.c. However, elution of this radioactivity followed by acetylation and rechromatography, as described in the Methods and Materials section, showed that none of it was due to 26-hydroxycholesterol.

Discussion

The present work confirms the observation of Fredrickson & Ono (1956) and of Danielsson (1961) that radioactive 26-hydroxycholesterol is formed from [^{14}C]cholesterol incubated with liver mitochondria. Radioactive 25-hydroxycholesterol is also formed in the incubation mixture but is readily separated from 26-hydroxycholesterol by t.l.c. after acetylation. Mendelsohn & Mendelsohn (1968) observed the formation of 25-hydroxycholesterol during incubation of [^{14}C]cholesterol with liver mitochondria, but were unable to demonstrate the formation of 26-hydroxycholesterol. We can suggest no explanation for this. However, it should be noted that an initial hydroxylation at C-26 is consistent with the formation of propionate (or propionyl-CoA) as the product of cleavage of the cholesterol side chain by liver mitochondria (Mitropoulos & Myant, 1965*a*,*b*), whereas hydroxylation at C-25 could not lead to propionate as the immediate product of side-chain cleavage.

The formation of some radioactive 26-hydroxycholesterol by non-enzymic mechanisms during the incubation cannot be excluded, but was probably of little quantitative significance since no 26-hydroxycholesterol was detectable in the boiled preparations assayed by the [^{3}H]acetic anhydride method. In keeping with this, radioactivity recovered from the 26-hydroxycholesterol band on t.l.c. of boiled preparations decreased progressively on repeated crystallization. The radioactivity recorded as 26-hydroxycholesterol in the boiled samples in Tables 1 and 2 was probably due largely to acetylated radioactive material with R_F 0.40, rather than to cholest-5-ene-3β,26-diol diacetate.

In addition to radioactive 26-hydroxycholesterol, more polar radioactive compounds are formed during the incubation and appear in the aqueous layer of the chloroform–methanol extract and in the polar fraction of the chloroform layer. As shown elsewhere (Mitropoulos & Myant, 1967*a*), these polar compounds include 3β-hydroxychol-5-enoic acid, lithocholic acid, chenodeoxycholic acid and α- and β-muricholic acid. The observation in Expt. 1 that addition of 26-hydroxycholesterol increased the accumulation of 26-hydroxy[^{14}C]cholesterol, and decreased the accumulation of radioactivity in the aqueous layer and in the monohydroxy acids, suggests that 26-hydroxycholesterol is an intermediate in the conversion of cholesterol into at least some of the bile acids formed in mitochondrial suspensions. Diminished production of $^{14}CO_2$ from [26-^{14}C]cholesterol in the presence of 26-hydroxycholesterol would also

be expected if 26-hydroxycholesterol is an intermediate in the cleavage of the cholesterol side chain leading to the formation of propionate and a C_{24} bile acid.

The results of Expt. 1, though suggestive, do not prove that bile acids were formed from cholesterol via 26-hydroxycholesterol, since the fall in bile acid radioactivity in the presence of 26-hydroxycholesterol could be due to inhibition of enzymes responsible for the formation of bile acids. However, the results of Expts. 2 and 3, in which addition of 26-hydroxycholesterol led to an increase in the mass of 3β-hydroxychol-5-enoic acid produced and to a fall in its specific radioactivity, provide strong evidence that 26-hydroxycholesterol is an intermediate in the formation of this bile acid. Since we have shown elsewhere that rat liver mitochondria convert 3β-hydroxychol-5-enoic acid into lithocholic acid, chenodeoxycholic acid and β-muricholic acid (Mitropoulos & Myant, 1967*b*), it is reasonable to suppose that these acids are also formed via 26-hydroxycholesterol by liver mitochondria. This would explain the observation of Wachtel *et al.* (1968) that 26-hydroxy[^{3}H]cholesterol is converted into radioactive 3β-hydroxychol-5-enoic acid, chenodeoxycholic acid and muricholic acid in bile-fistulated rats. The finding that in the absence of exogenous 26-hydroxycholesterol the specific radioactivity of 26-hydroxycholesterol was higher than that of 3β-hydroxychol-5-enoic acid would be expected if 26-hydroxycholesterol is an intermediate in the formation of this acid.

The absence of a significant effect of added 26-hydroxycholesterol on the accumulation of radioactivity in 3β-hydroxychol-5-enoic acid in Expts. 2 and 3 suggests that in these experiments the added 26-hydroxycholesterol was not sufficient to saturate the cholesterol 26-hydroxylase in the mitochondria. This could have been due to incomplete mixing of the added 26-hydroxycholesterol with the pool of 26-hydroxycholesterol derived from cholesterol and from which bile acids are formed in the mitochondrial suspension. This would explain why, in the presence of added 26-hydroxycholesterol, the specific radioactivity of the 3β-hydroxychol-5-enoic acid formed was higher than that of the 26-hydroxycholesterol isolated at the end of the incubation.

The [4-^{14}C]cholesterol added to the incubation mixture contained 20nmol of cholesterol of specific radioactivity 1.1×10^5d.p.m./nmol, but the specific radioactivity of the 26-hydroxy[^{14}C]cholesterol isolated from the incubation mixture in Expts. 2 and 3 was between 6.5×10^3 and 8.2×10^3 d.p.m./nmol (mean, 7.3×10^3d.p.m./nmol) in the absence of added 26-hydroxycholesterol. This shows that the 26-hydroxy[^{14}C]cholesterol was derived from a pool of cholesterol larger than the mass of exogenous cholesterol added.

Cronholm & Johansson (1970) have shown that the introduction of a 26-hydroxyl group into 5β-cholestane-3α,7α,12α-triol is catalysed by rat-liver microsomes in the presence of NADPH, but not by rat liver mitochondria. The enzyme system responsible for this hydroxylation is probably different from that responsible for the mitochondrial 26-hydroxylation of cholesterol, since cytochrome *P*-450, which has not been shown to be present in liver mitochondria, is required for the microsomal hydroxylation of 5β-cholestane-3α,7α,12α-triol (Cronholm & Johansson, 1970). Moreover, in agreement with the present work, H. Danielsson (personal communication) has shown that suspensions of rat liver microsomes do not catalyse the hydroxylation of the side chain of cholesterol.

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