Mechanism of Formation of Bile Acids from Cholesterol: Oxidation of 5β-Cholestane-3α,7α,12α-triol and Formation of Propionic Acid from the Side Chain by Rat Liver Mitochondria*

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It has been established by Bergström, Pääbo, and Rumpf (1, 2) that the formation of cholic acid from cholesterol depends upon a complex series of enzymatic reactions involving initial hydroxylation of the nucleus followed by cleavage of the isooctyl side chain.

The fact that 5β-cholestane-3α,7α,12α-triol is efficiently converted *in vivo* to cholic acid (2) lends considerable support to the notion that 5β-cholestane-3α,7α,12α-triol is probably a normal and obligatory intermediate in the conversion of cholesterol to cholic acid.

Previous efforts in this laboratory to investigate the fate of the 3 terminal carbon atoms of the cholesterol side chain have been hampered by the slow rate of hydroxylation of cholesterol by cell-free preparations of rat liver (3). Following Bergstrom's work (2), Briggs, Whitehouse, and Staple (4) demonstrated that 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid is rapidly converted to cholic acid by rat liver mitochondria.

The present investigation was designed to investigate the metabolic fate of the 3 terminal carbon atoms of the side chain of 5β-cholestane-3α,7α,12α-triol.

EXPERIMENTAL PROCEDURE

Materials

ATP (disodium salt), DPN, GSH and CoA were obtained from Sigma Chemical Company. 2-Propanol-1,3-C¹⁴ was purchased from Nuclear-Chicago Corporation.

5β-Cholestane-3α,7α,12α-triol-26,27-C¹⁴ was synthesized from triformylcholyl chloride and diisopropylcadmium-1,3-C¹⁴ according to the method of Staple and Whitehouse (5).

3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid, isolated from alligator bile, was kindly supplied by Dr. T. Briggs.

5β-Cholestane-3α,7α,12α,26-tetrol was obtained by reduction of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, isolated from alligator bile, with lithium aluminum hydride.

Propionyl-CoA was prepared from propionic anhydride and CoA according to the method of Simon and Shemin (6).

Methods

Fractionation of Rat Liver Homogenates—Washed mitochondria were prepared from livers of 6- to 8-week-old male Wistar rats by the procedure of Whitehouse, Staple, and Gurin (3). Microsomes were obtained from the supernatant suspension of the first centrifugation at 8500 × g by centrifugation for 60 minutes at 105,000 × g. The supernatant fraction from this last centrifugation was used in incubations and will be referred to as the supernatant fluid.

Incubations with THC-26,27-C¹⁴—The substrate, THC-26,27-C¹⁴,[1] was dissolved in methanol, and the specified aliquots were pipetted into individual 50-ml Erlenmeyer flasks. After addition of a solution of 4 mg of Tween 20[2] in methanol, the solvent was evaporated in a stream of nitrogen at 40–50°. To the warm residue were added 5 ml of 0.25 M Tris hydrochloride buffer, pH 8.5, and the mixture was emulsified by shaking the contents for 1 minute. After cooling, 1 ml of a solution containing ATP (25 mg), DPN (5 mg), GSH (15 mg), nicotinamide (36 mg), magnesium chloride hexahydrate (8 mg), and trisodium citrate dihydrate (22 mg) was added to each flask. Finally, a suspension of mitochondria equivalent to 4 g of rat liver, supernatant fluid equivalent to 1.5 g of liver, and a sufficient quantity of 10% aqueous sucrose solution were added to give a final volume of 12.5 ml. Incubations were conducted aerobically for specified lengths of time at 37° with constant mechanical agitation. Incubations were terminated by the addition of 40 ml of 95% ethanol. When the formation of carbon dioxide from THC-26,27-C¹⁴ was to be measured, 125-ml Warburg flasks were used. The carbon dioxide trapped in the alkali of the center wells was precipitated as barium carbonate, and the radioactivity was determined in a liquid scintillation counter as described previously (3).

Isolation of Labeled 26-OH-THC and THCA from Incubations with THC-26,27-C¹⁴—The precipitate formed upon addition of ethanol to the incubation mixtures was removed by centrifuga-

[1] The abbreviations used are: THC, 5β-cholestane-3α,7α,12α-triol (3α,7α,12α-trihydroxycoprostane); THCA, 3α,7α,12α-tri-hydroxy-5β-cholestan-26-oic acid (3α,7α,12α-trihydroxyco-prostanoic acid); 26-OH-THC, 5β-cholestane-3α,7α,12α-tetrol (3α,7α,12α,26-tetrahydroxycoprostane).

[2] Atlas Powder Company, Wilmington, Del.

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tion, and the supernatant solution was evaporated to dryness in a flash-evaporator at 25–30°. The residue was suspended in a small volume (3 to 4 ml) of water, acidified with 10 N sulfuric acid to pH 1 to 2, mixed thoroughly with Celite 535 which had been purified by washing with water, methanol, and ether, and then air-dried. The amount of the absorbent used was 2 g of Celite for 1 ml of the sample. The mixture was packed firmly into a column (internal diameter, 18 mm) and eluted with 150 ml of ether. The ether eluates were combined and evaporated to dryness, and the residue was subjected to paper chromatography according to the procedure of Sjövall (7). The solvent system employed was 70% aqueous acetic acid as the stationary phase and isopropyl ether-heptane (6:4) as the mobile phase. After application of the ether-soluble materials to Whatman No. 1 paper, the paper was dipped into 70% aqueous acetic acid, blotted between two sheets of Whatman No. 3 mm paper, and partly dried in air for 10 to 12 minutes. The paper was then placed into a chromatographic jar saturated with vapors of both phases. After equilibration for 1 hour, the descending chromatograms were developed for either 3 to 4 hours or 10 hours. The developed chromatograms were air-dried, and the distribution of radioactivity was determined by scanning with a Nuclear-Chicago Automatic Scanner. The radioactive peaks were eluted from the paper with methanol and purified by column chromatography.

The labeled THCA eluted from the papers was subjected to partition column chromatography as described by Mosbach, Zomzely, and Kendall (8). Celite 545, 4 g, was mixed with 5 ml of 70% aqueous acetic acid and packed in a column (0.9 × 18 cm). After 100 ml of petroleum ether (boiling point, 30–60°) had been percolated through the column, the sample, dissolved in two drops of glacial acetic acid and mixed with 0.2 g of Celite 545, was placed on the top of the column. Elution was effected with 45 ml of petroleum ether followed by 150 ml of 40% isopropyl ether in petroleum ether. Both eluants had previously been saturated with 70% aqueous acetic acid at the temperature at which the chromatograms were developed (24.5°).

The labeled 26-OH-THC eluted from the papers was further purified by reversed phase partition chromatography on a Super-Cel column. Hyflo Super-Cel was rendered hydrophobic according to the procedure described by Howard and Martin (9). The column was developed with a solvent system described by Bergström, Bridgwater, and Gloor (10), the stationary phase being 4 ml of chloroform-heptane (9:1) on 4.5 g of hydrophobic Super-Cel, and the moving phase, 50% aqueous methanol. Both phases were equilibrated with each other at the temperature at which the chromatograms were developed (24.5°).

Isolation of Labeled Propionic Acid from Incubations with THC-26,27-C¹⁴ and Carrier Propionic Acid—Incubation conditions and cofactors were the same as described for THC-26,27-C¹⁴, except for the added potassium propionate (1.5 mg per flask). Incubations were terminated by heating the flasks for 1 to 2 minutes on a boiling water bath. The resulting precipitate was removed by centrifugation. To the supernatant solution were added 2 mg of potassium propionate and sufficient 5 N sulfuric acid to attain a pH of 1 to 2. The solution was then steam-distilled until 150 to 175 ml of the distillate were collected. The steam distillate was neutralized with 0.1 N sodium hydroxide (phenolphthalein), and small aliquots of the solution were evaporated and subsequently counted in a Geiger-Müller counter. The major portion of the neutralized steam distillate was flash-

evaporated to dryness, and the residue was chromatographed on a Celite 535 column according to the procedure of Swim and Utter (11), the stationary phase being 7.5 ml of 0.2 N sulfuric acid on 15 g of Celite 535, and the moving phase, chloroform saturated with 0.2 N sulfuric acid. To each 10-ml effluent fraction were added 5 ml of carbon dioxide-free water, and the individual fractions were titrated with 0.01 N sodium hydroxide to the phenol red end point. Small aliquots of the neutralized solution were used for estimation of radioactivity. The fractions found to contain the labeled propionate were flash-evaporated to dryness, and the residues were used for the preparation of the propionic acid derivatives as well as for the chemical degradation of the propionic acid.

Incubations with 26-OH-THC-26,27-C¹⁴ and Carrier Propionyl-CoA—Incubations were carried out with microsomes corresponding to 1.25 g of rat liver and the supernatant fluid corresponding to 0.5 g of liver. Other additions to each incubation flask were 26-OH-THC-26,27-C¹⁴ (4000 c. p. m.) emulsified with 1.5 mg of Tween 20 and 1.5 ml of 0.25 M phosphate buffer, pH 8.0, 0.5 mg of potassium propionate, 7.3 mg of ATP, 1.3 mg of DPN, 3.7 mg of GSH, 8.8 mg of nicotinamide, 2.0 mg of magnesium chloride hexahydrate, 5.5 mg of trisodium citrate dihydrate, and 11.8 mg of tetrasodium pyrophosphate. The final volume of the incubation mixtures was 3.6 ml. The mixtures were allowed to incubate for 10 minutes, at which time 5 mg of freshly prepared propionyl-CoA were added to each flask and incubation continued for another 10 minutes. After a total time of 20 minutes, the incubation flasks were divided into three groups according to the objective of the further work-up: Group I, assay of the labeled propionate in the steam distillate; Group II, isolation of propionyl hydroxamate from the incubation mixtures; and Group III, isolation and identification of propionyl-CoA.

For the formation of propionyl hydroxamate in the incubation mixtures, the method described by Stadtman and Barker (12) was followed. At the end of the incubation period, the pH of the medium was adjusted to 5.5 to 6.0 with 1 N hydrochloric acid. Hydroxylamine reagent (4 ml), freshly prepared from equal volumes of 3.5 N sodium hydroxide solution and aqueous hydroxylamine hydrochloride (28%), was added to each flask. After these mixtures had stood at room temperature for 10 minutes, 100 ml of ethanol were added, and the resulting precipitate was removed by centrifugation. The supernatant solution was flash-evaporated to dryness, the dry residue was extracted with three 2-ml portions of absolute ethanol, and the extracts were then combined and evaporated under a slow stream of nitrogen to a volume of 0.3 ml. The precipitated solid was removed by centrifugation, and the supernatant solution was subjected to paper chromatography. Descending chromatograms were developed for 5 to 6 hours in an octyl alcohol-formic acid-water (75:25:75, volume for volume) solvent system (13). The developed chromatograms were dried overnight at room temperature; a narrow strip was then cut lengthwise from the chromatogram paper and dipped into a ferric chloride solution prepared from 5 g of ferric chloride hexahydrate in 100 ml of 0.1 N hydrochloric acid in 95% alcohol (13). The area corresponding to propionyl hydroxamate (R_F 0.28) was eluted with ethanol, and the eluates were evaporated under a slow stream of nitrogen to a volume of 0.2 to 0.3 ml. This solution was then rechromatographed on paper with an n-butanol-acetic acid-water (4:1:5, volume for volume) solvent system (13). A narrow strip was cut lengthwise from the developed and dried

paper chromatogram and dipped into the ferric chloride reagent described above. The area corresponding to propionyl hydroxamate (R_F 0.67) was eluted from paper with ethanol, and the evaporated eluates were counted in a Geiger-Müller counter. The area in the immediate vicinity of the propionyl hydroxamate was also eluted and checked for radioactivity.

A modified procedure, based on the method of Lynen, Reichert, and Rueff (14) for the isolation of acetyl-CoA from yeast was used for the isolation of propionyl-CoA. After incubation, each flask was treated with 1.2 g of ammonium sulfate, and the mixture was extracted with three 1-g portions of phenol. The phenolic extracts were combined, an equal volume of ether was added, and the resulting mixture was extracted with five 1-ml portions of water. The aqueous extracts were combined and extracted with 2 ml of ether, and the remaining aqueous layer was flash-evaporated at 20° to a volume of 0.3 ml. This solution was then chromatographed on Whatman No. 1 paper with an isopropanol-pyridine-water (1:1:1, volume for volume) solvent system (15). The developed chromatograms were dried in air, and a portion of the paper was dipped into the nitroprusside reagent of Toennies and Kolb (16) and then sprayed with methanolic sodium hydroxide solution. The area of the remaining paper which corresponded to propionyl-CoA (R_F 0.34) was cut out and sprayed with freshly prepared hydroxylamine reagent. After the paper strip was air-dried for 20 minutes, the resulting hydroxamate was eluted from paper with ethanol. The eluates were concentrated to a volume of 0.2 to 0.3 ml under a slow stream of nitrogen, and the concentrate was chromatographed in the two solvent systems described above for the isolation of propionyl hydroxamate. The propionyl hydroxamate spot was eluted from the paper and assayed for radioactivity.

RESULTS

Rat liver mitochondria, supplemented with supernatant fluid, ATP, DPN, GSH, nicotinamide, magnesium chloride, and citrate, were observed to oxidize THC-26,27-C^{14} into more polar products which could be separated by paper chromatography. Fig. 1 shows the distribution of radioactivity when ether-soluble materials from incubation mixtures were chromatographed for 4 hours. Peak II (R_F 0.74) was found to be the unchanged THC-26,27-C^{14}, whereas Peak I (R_F 0.36) appeared to be THCA. However, when Peak I was eluted from the paper and rechromatographed in the same solvent system for 10 hours, two radioactive peaks, IA and IB, were observed on scanning. Peak IB had a mobility identical with that of THCA in the same system. After separation of Peaks IA and IB by paper chromatography, Peak IB was further purified by partition chromatography on a Celite 545 column (Fig. 2). The effluent fractions of 75 to 100 ml yielded a colorless crystalline material which melted, after several recrystallizations from ether, at 172–173°. This compound gave no melting point depression on admixture with an authentic sample of THCA (melting point, 172–173°) isolated from alligator bile. Also, the infrared spectra of both compounds were identical.

To determine whether THCA (Peak IB) and the substance under the Peak IA were interconvertible, separate incubations with both of these compounds were carried out. When mitochondria were supplemented with supernatant fluid, both THCA and the material represented in Peak IA were degraded to an extent at which no radioactivity could be detected when the individual paper chromatograms were scanned (Table I). However, incubations without the supernatant fluid demonstrated that the substance represented by Peak IA could be converted into THCA. The omission of any of the individual cofactors resulted in a decreased conversion of the substance into THCA. The omission of DPN was most effective in preventing this conversion (Table II). The fact that conversion of the unknown Peak IA to THCA was greatly suppressed when DPN was omitted furnished us with a method for the isolation of the unknown metabolite. The ether extracts obtained from a large number of incubations without DPN were chromatographed on paper in the isopropyl ether-heptane-acetic acid solvent system. Peak IA was eluted from the papers with methanol and chro-
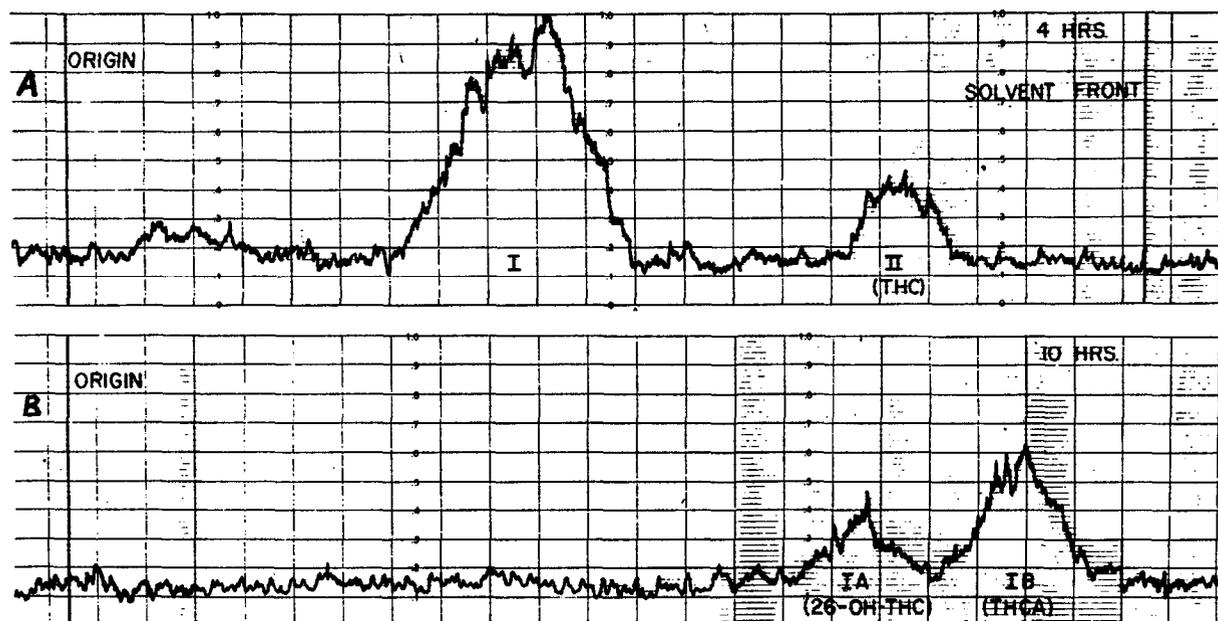

Fig. 1. Isotope distribution on paper chromatograms of ether-soluble materials from incubations with THC-26,27-C^{14}. A, chromatogram of ether extracts from 1½ hour-incubation; B, Peak I from Chromatogram A rechromatographed for 10 hours.
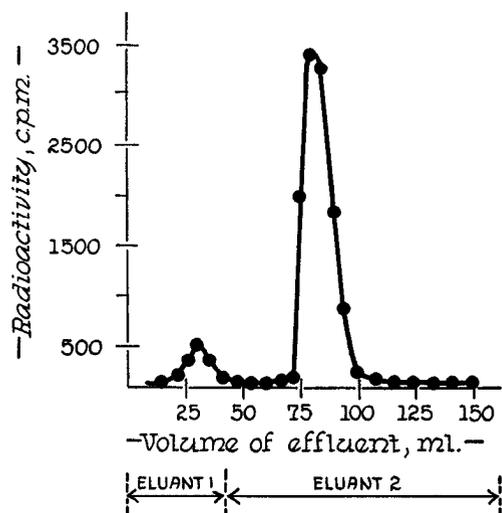
Fig. 2. Partition chromatography of Peak IB on Celite 545

matographed on a Super-Cel column with the chloroform-heptane-50% aqueous methanol solvent system. The effluent fractions, between 50 and 75 ml, furnished crystalline material which, after recrystallization from aqueous methanol, melted at 203–204°. The infrared spectrum of this compound showed no carbonyl function. Inferring that the unknown solid was 26-OH-THC, we synthesized an authentic sample of this steroidal alcohol from THCA which had been isolated from alligator bile by reduction with lithium aluminum hydride. The melting point of a mixture of crystalline material from Peak IA with the synthetic 26-OH-THC was not depressed. The infrared spectra of the two compounds were identical. It is of interest that the enzymatic reaction from 26-OH-THC to THCA could not be reversed.

The fact that terminally labeled 26-OH-THC and THCA could be metabolized by rat liver mitochondria fortified with supernatant fluid, to a degree where no detectable radioactivity could be recovered in any steroid fraction, suggested that this enzyme system was rapidly attacking the terminal carbon atoms of the side chain. It was striking also (see Table I) that very little simultaneous evolution of labeled carbon dioxide was observed. These results suggested that cleavage of the side chain to form a 3 carbon fragment occurs before evolution of carbon dioxide. Since previous attempts to demonstrate a significant production of lactic and pyruvic acids were unsuccessful, experiments were designed to determine whether the primary cleavage product was propionic acid.

Incubations of THC-26,27-C^{14} with carrier propionic acid resulted in a recovery of approximately 26% of the isotope in the steam-distillable fractions, which, after column chromatography on Celite 535 (Fig. 3), yielded labeled propionic acid. Approximately 80% of the radioactivity present initially in the steam distillate was recovered in the propionic acid peak. When, in addition to the previously described cofactors, CoA was added to the incubation medium, 42% of the substrate isotope was recovered in the steam distillate, of which, again, approximately 80% was found to be present in the propionic acid peak (Fig. 3).

Two derivatives of the isolated propionic acid were prepared: the *p*-toluidide (melting point 123–124°) and the *S*-benzyl-thiuronium salt (melting point, 148°), which were then recrystallized to constant specific activity. Thus, 96 and 99% of

TABLE I

Isotope recovery from incubations with THCA-26,27-C^{14} and with material from Peak IA

Ether-soluble materials from the incubation mixtures were chromatographed in isopropyl ether-heptane-acetic acid solvent system for 10 hours. Per cent conversions were estimated from the areas under the scanning curves; controls with boiled enzyme were taken as 100%.

Cell Fraction	Amount of Peak IA	Amount of Peak IB (THCA)	Radioactivity of BaCO·

A. Substrate, THCA-26,27-C^{14} (8000 c. p. m.); usual cofactors; incubation time, 1½ hours.

	%	%	c.p.m.
Boiled enzyme	0	100	0
Mitochondria	0	80	0
Supernatant	0	100	0
Mitochondria + supernatant	0	0	130

B. Substrate, material from peak IA (8000 c. p. m.); usual cofactors; incubation time, 1½ hours.

Boiled enzyme	100	0	0
Mitochondria	50	20	0
Supernatant	70	30	0
Mitochondria + supernatant	0	0	90

TABLE II

Incubations with THCA-26,27-C^{14} and material from Peak IA in absence of various cofactors

Complete system contained mitochondria, supernatant fluid, ATP, DPN, GSH, nicotinamide, magnesium chloride, and citrate; incubation time was 1½ hours. Ether-soluble materials from the incubation mixtures were chromatographed on paper in isopropyl ether-heptane-acetic acid solvent system for 10 hours. Per cent conversions were estimated from the areas under the scanning curves; controls with boiled enzyme were taken as 100%.

	Amount of Peak IA	Amount of Peak IB (THCA)

A. Substrate, THCA-26,27-C^{14} (9000 c. p. m.)

	%	%
Complete system	0	0
− DPN, − nicotinamide	0	65
− ATP	0	95
− GSH	0	80
− Magnesium chloride	0	85
− Citrate	0	65
Boiled enzyme	0	100

B. Substrate, material from Peak IA (9000 c.p.m.)

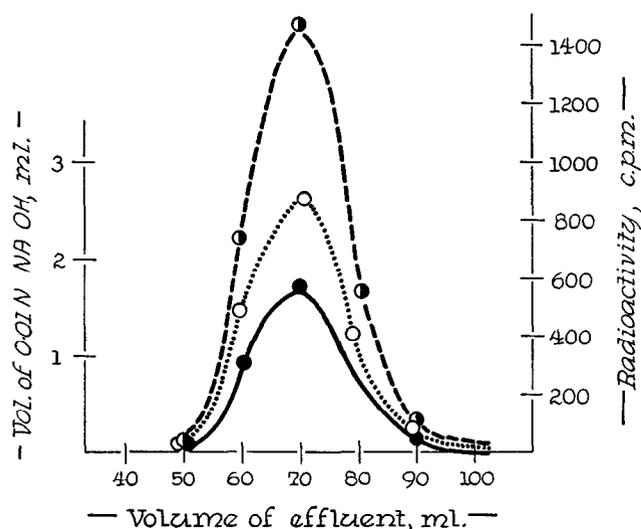|		
Complete system	0	0
− DPN, − nicotinamide	80	0
− ATP	50	35
− GSH	40	20
− Magnesium chloride	45	35
− Citrate	50	10
Boiled enzyme	100	0

Fig. 3. Chromatography of the steam-distillable fractions from 3-hour incubations with carrier propionate and THC-26,27-C^{14} (0.11 μmole, 8500 c.p.m.) on Celite 535 column. ●——●, titration curve of propionic acid; O····O, radioactivity curve with no CoA added; ◖---◖, radioactivity curve with CoA added.

TABLE III

Distribution of C^{14} in propionic acid isolated from incubations with THC-26,27-C^{14} and carrier propionate

Propionic acid component	Radioactivity
	c. p. m./μmole
Sodium propionate	47,000
BaCO₃ derived from:	
Carbon 1	22,660
Carbon 2	98
Carbon 3	19,300

the theoretical number of counts were accounted for in the *p*-toluidide and the *S*-benzylthiuronium salt, respectively.

Degradation of the isolated propionic acid according to Phares (17) yielded carbon dioxide and ethylamine; the amine was oxidized with permanganate, and the resulting acetic acid was submitted to the same degradative procedure. The results demonstrated that the propionic acid derived from THC-26,27-C^{14} was labeled at carbons 1 and 3, whereas carbon 2 contained essentially no C^{14} (Table III). Carbons 1 and 3 were labeled to an equal degree despite a small discrepancy in the values, which can be ascribed to a technical error.

That propionyl-CoA might be the cleavage product was suggested by the observation that addition of CoA to the incubation medium increased the formation of labeled propionate from THC-26,27-C^{14}. Hence, incubations with 26-OH-THC-26,27-C^{14} and carrier propionyl-CoA were performed to determine whether any of the substrate label appeared in the recovered propionyl-CoA. However, none of the added carrier propionyl-CoA could be recovered from incubations carried out under the usual conditions; this was ascribed to very active deacylase activity. Consequently, incubations were run in phosphate buffer, pH 8.0, with added tetrasodium pyrophosphate to diminish deacylase activity. Under these conditions, a small

amount of carrier propionyl-CoA could be isolated, provided that the incubation period was shortened to 10 minutes. Paper chromatography of the phenol-soluble materials was followed by the treatment of the propionyl-CoA spot with hydroxylamine reagent and subsequent elution of the propionyl hydroxamate from the paper. To insure the purity of the propionyl hydroxamate, paper chromatograms were developed in two different solvent systems; the propionyl hydroxamate area was eluted and the eluates were assayed for radioactivity. Thus, 10% of the radioactivity present in the steam distillate (determined in a control flask) was recovered in the propionyl hydroxamate. However, when hydroxylamine reagent was added directly after the incubation, a much larger recovery of radioactivity (29%) could be obtained in the propionyl hydroxamate.

DISCUSSION

The formation of bile acid conjugates by the liver is known to be enzyme-catalyzed (18–20). The activation of cholic acid for this process has been studied by Siperstein and Murray (20), who demonstrated that cholic acid is converted to cholyl-CoA in the presence of CoA, ATP, magnesium chloride, and an enzyme present in the microsomes of guinea pig liver. The subsequent conjugation of cholyl-CoA with amino acids is catalyzed by a "transferase" present in the supernatant fraction of the liver.

The results of the present investigation indicate that cholyl-CoA is probably formed by a thiolytic cleavage of the cholesterol side chain. The recovery of isotope in propionyl-CoA from incubations with THC-26,27-C^{14}, coupled with the observation that addition of CoA to the incubation medium stimulated the formation of labeled propionate, suggests that both propionyl-CoA and cholyl-CoA are probably formed by a thiolytic cleavage of 3α,7α,12α-trihydroxy-24-keto-5β-cholestan-26-oyl-CoA (Fig. 4). This thiolytic cleavage is analogous to the corresponding sequence of reactions involved in the oxidation of methylbutyric acid (21). Since THC used in the present investigation was labeled only in the isopropyl moiety of the side chain, it was not possible to demonstrate the formation of cholic acid (cholyl-CoA) from THCA. However, the conversion of THCA-4-C^{14} to cholic acid in rat liver mitochondria has been previously demonstrated by Briggs, Whitehouse, and Staple (4).

The identification of propionic acid-1,3-C^{14} as the major cleavage product derived from the side chain of THC-26,27-C^{14} is in agreement with previous observations on studies of cholesterol side chain oxidation. Thus, it was found that only 10 to 15% of the isotope of cholesterol-26-C^{14} was oxidized to carbon dioxide after prolonged incubation periods, and that carbon dioxide production was suppressed by reagents known to inhibit the functioning of the tricarboxylic acid cycle (3). These results suggested that the carbon dioxide derived from C-26 of cholesterol could have been produced by the oxidation of some precursor of the tricarboxylic acid cycle. Furthermore, Briggs, Whitehouse, and Staple (4) observed that the rapid formation of cholic acid from THCA-4-C^{14} in rat liver mitochondria during the first 2 hours of incubation was accompanied by little or no labeled carbon dioxide. In these experiments, THCA-26,27-C^{14} was also present. The formation of labeled carbon dioxide occurred subsequent to the 2-hour lag period.

While the present study was in progress, oxidation of THC by mouse and rat liver mitochondria was reported by Danielsson (22). He observed that, whereas mouse liver mitochondria

3α, 7α, 12α - TRIHYDROXY-
5β - CHOLESTAN - 26 - OYL CoA
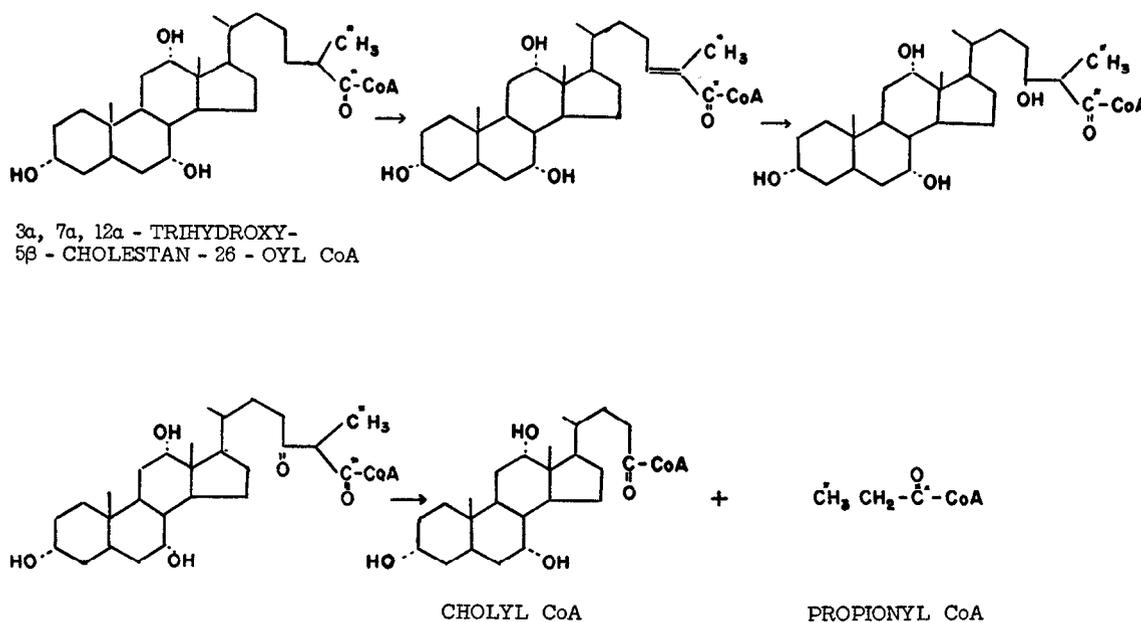

CHOLYL CoA   PROPIONYL CoA

FIG. 4. Proposed mechanism for formation of cholyl-CoA and propionyl-CoA

oxidize THC to both 26-OH-THC and THCA, only 26-OH-THC is formed by rat liver mitochondria. The difference between the results obtained by Danielsson with rat liver mitochondria and the findings of the present study may be due to the omission of cofactors by Danielsson.

It appears, therefore, that the oxidative scheme postulated by Fieser and Fieser (23) for the conversion of THC to THCA via 26-OH-THC has been substantiated by both the present investigation and the findings of Danielsson. Conversion of THCA to cholic acid both *in vivo* (24) and *in vitro* (4, 10) has been previously demonstrated. Attempts to demonstrate the conversion of cholesterol to any of the above-mentioned intermediates or to cholic acid *in vitro* have so far been unsuccessful. This can probably be ascribed to the omission of some necessary cofactors, or to the destruction of the enzyme systems necessary for hydroxylation of the steroid nucleus, which results from the process of preparing cell-free preparations. However, conversion of cholesterol to THCA in the alligator (4) suggests that THCA is a direct intermediate in the biosynthesis of cholic acid.

SUMMARY

Rat liver mitochondria, supplemented with 105,500 × *g* supernatant fluid, adenosine triphosphate, diphosphopyridine nucleotide, glutathione, nicotinamide, magnesium chloride, and citrate, can oxidize 5β-cholestane-3α,7α,12α-triol-26,27-C^{14} to 5β-cholestane-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. In the absence of diphosphopyridine nucleotide, the main product is 5β-cholestane-3α,7α,12α,26-tetrol. Both products were isolated by paper and column chromatography and were characterized by infrared spectra as well as by mixed melting points with authentic samples.

The enzyme system also cleaves the side chain of 5β-cholestane-3α,7α,12α-triol to propionic acid. Carrier experiments revealed the incorporation of 21% of the substrate isotope into propionic acid, which was characterized by preparation of *p*-toluidide and

S-benzylthiuronium salt. The addition of coenzyme A stimulated this conversion. Degradation of the isolated propionic acid demonstrated that only carbons 1 and 3 contained C^{14}.

Incubation of 5β-cholestane-3α,7α,12α,26-tetrol-26,27-C^{14} with carrier propionyl coenzyme A permitted the recovery of labeled propionyl coenzyme A.

It is proposed that propionyl coenzyme A is the major 3 carbon fragment produced in the conversion of cholesterol to the bile acids. Present evidence suggests that cholyl coenzyme A and propionyl coenzyme A are produced by thiolytic cleavage of a postulated intermediate, 3α,7α,12α-trihydroxy-24-keto-5β-cholestan-26-oyl coenzyme A.

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