Bile Acid Synthesis in Man: Metabolism of 7α-Hydroxycholesterol-^{14}C and 26-Hydroxycholesterol-^{3}H

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ABSTRACT The pathways of bile acid synthesis in man were evaluated by studying the metabolism of 7α-hydroxycholesterol-4-^{14}C and 26-hydroxycholesterol-16, 22-^{3}H administered parenterally to individuals requiring external biliary drainage. Techniques for the identification of metabolites were thin-layer chromatography, column chromatography, gas-liquid chromatography with stream splitting, and crystallization to constant specific activity. It was found that both compounds were rapidly metabolized to bile acids and excreted in bile. Of the total radioactivity recovered in bile as bile acids, 87% of the 26-hydroxycholesterol-^{3}H and 90% of the 7α-hydroxycholesterol-^{14}C was found to be metabolized to both chenodeoxycholate and cholate. Compared to 7α-hydroxycholesterol, a greater proportion of 26-hydroxycholesterol was found to be metabolized to chenodeoxycholate.

These findings indicate that both 7α-hydroxycholesterol and 26-hydroxycholesterol can be intermediates in the metabolism of cholesterol to bile acids in man. The observation that conversion to cholate takes place less readily after C-26 hydroxylation is consistent with previous findings in other species.

INTRODUCTION

It is recognized that the liver cell possesses enzymes that metabolize cholesterol [1] to chenodeoxycholate and cholate, the primary bile acids of man. Although none of the intermediates in the pathways have been isolated directly from liver, studies of the metabolic fate of various possible intermediates have led to the proposal of a logical sequence of reactions. These studies have been summarized recently (1, 2) and conclude that 7α-hydroxycholesterol is a precursor of both chenodeoxycholate and cholate and that 26-hydroxycholesterol is a precursor of chenodeoxycholate (1, 3). Using labeled 26-hydroxycholesterol of high specific activity, evidence has been obtained in the hamster and rat (4, 5) that 26-hydroxycholesterol is also metabolized to cholate. With the exception of one study using in vitro preparations of human liver (6), little information is available concerning the possible pathways in man. We have therefore evaluated the role of 7α-hydroxycholesterol and 26-hydroxycholesterol in the biosynthesis of bile acids in man, and found that both compounds are metabolized to primary bile acids and therefore can be considered intermediates.

METHODS

Patients. Seven patients requiring external biliary drainage were studied. Six patients had surgery for cholelithiasis and one patient had a stricture of the common duct. None of the patients had a total bile fistula. Studies were begun 3–25 days after surgery.

Materials. Synthesis of 7α-hydroxycholesterol from cholesterol was according to the method of Starka (7). Cholesterol-4-^{14}C purchased from New England Nuclear Corp., Boston, Mass. was used for the preparation of 7α-hydroxycholesterol-4-^{14}C. The synthesis of 26-hydroxycholesterol-16, 22-^{3}H has been described in detail previously (4). Only crystalline compounds giving a single zone of mass and radioactivity by thin-layer chromatography were used. Bile acids used as standards for thin-layer and gas-liquid chromatography were purchased from Supelco, Inc., Bellefonte, Pa.

Analytical procedures. Bile samples were reduced in volume with a freeze-dry apparatus, after which sufficient

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[1] Systematic names of steroids referred to in this paper by their trivial names are: cholesterol, cholest-5-en-3β-ol; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 26-hydroxycholesterol, cholest-5-en-3β,26-diol; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

NaOH and EDTA solutions were added to give a final concentration of 1.25 N and 2.5 mg/ml respectively. Hydrolysis was then carried out at one atmosphere (121°C) for 3 hr. Ethyl acetate was used for extraction of cholesterol and other neutral lipids before acidification and for extraction of bile acids after acidification to pH 1 with HCl. The bile acids were methylated as described previously (8) and further purified by thin-layer chromatography using Silica Gel G and by column chromatography with neutral alumina, activity grade V. Crystallization of the purified fractions was done by methods previously described (4).

For gas-liquid chromatography (GLC)[2] either the acetates, using acetic anhydride and perchloric acid at 0°C as described by Roovers, Evrard, and Vanderhaeghe (9), or the trifluoroacetates (10), of the bile acid methyl esters were prepared. The keto bile acid 3α-7α-dihydroxy-12-keto-5β-cholanoic acid, purchased from Mann Research Labs, Inc., New York, was used as an internal standard. Peaks were quantitated by an electronic integrator. A Hewlett-Packard Model 402 (Hewlitt-Packard Co., Palo Alto, Calif.) equipped with a flame detector and a Barber-Coleman stream splitter (Barber-Coleman Company, Rockford, Ill. was used. Analytical separations were carried out on 4-ft columns (3 mm inner diameter) packed with 2% OV-210 (Applied Science Laboratories, Inc., State College, Pa.) and with a carrier flow rate of 70 ml/min of helium, as previously described (8). For stream splitting a column with an 8 mm outer diameter was used with a carrier flow rate of 300 ml/min.

Repeated portions of the same sample were injected to obtain sufficient radioactivity without overloading the column. The radioactive effluent was collected at 1 min intervals in capillary glass tubes at room temperature. Each interval from successive injections was combined and crushed in a counting vial to which liquid scintillant (6 g 2,5-diphenyloxazole and 75 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene) was then added.

Radioactivity was measured by liquid scintillation spectrometry and was expressed in dpm after correction by internal standardization. For counting of the effluent obtained during GLC stream splitting studies, the efficiency was adjusted so that there was less than 2% crossover of ^{14}C and ^{3}H and less than 0.1% crossover of ^{3}H into ^{14}C. The results are expressed as cpm with no correction for background.

Experimental design. After the collection of control bile samples, an infusion of equimolar tracer amounts (300–600 μg) of 26-hydroxycholesterol-^{3}H (SA 13.3 μCi/μmole) and 7α-hydroxycholesterol-^{14}C (1.3 μCi/μmole) was given by vein over a 20 min period. One patient was given only 7α-hydroxycholesterol-^{14}C. The compounds were dissolved in 0.1 ml ethanol and added to 50 ml of 25% human serum albumin with thorough mixing before infusion. Bile samples were then collected at 2–4 hr intervals for the initial 8 hr, and then for longer periods up to 24 or 48 hr.

RESULTS

Recovery of radioactivity in bile. As might be expected in patients with partial biliary drainage, the recovery of radioactivity in bile was variable (Table I). Of the total radioactivity recovered in bile the greatest proportion was obtained within 24 hr and often the rate of excretion of both ^{3}H and ^{14}C was highest in the initial 4 hr period. In two paitents total recovery of each isotope was the same, while in one ^{3}H recovery exceeded ^{14}C recovery, and in three ^{3}H recovery was less than ^{14}C recovery.

TABLE I

Per Cent Radioactivity Recovered in Bile of Patients Given 26-Hydroxycholesterol-^{3}H and 7α-Hydroxycholesterol-^{14}C

Patient	Isotope	0–4 hr*	4–8 hr	8–24 hr	24–48 hr	Total
		% of administered dose				
J. A.‡	^{3}H	—	31	29	24	84
	^{14}C	—	33	33	24	90
M. Pi.	^{3}H	40	5	3	NC§	48
	^{14}C	72	9	5		86
J. K.	^{3}H	8	2	NC	NC	10
	^{14}C	22	6			28
M. Sa.	^{3}H	32	22	13	NC	67
	^{14}C	29	23	9		61
M. Sc.	^{3}H	—	—	55‖	NC	55
	^{14}C	—	—	46		46
S. B.	^{3}H	5	7	10	NC	22
	^{14}C	6	12	19		37
M. Pu.¶	^{14}C	36	16	11	2	65

* Hr after beginning of infusion.
‡ Bile collected 0–8 hours in this patient.
§ NC, bile not collected.
‖ Bile collected 0–24 hr.
¶ This patient given only 7α-hydroxycholesterol-^{14}C.

Identification of radioactive metabolites in bile. The bile sample from each patient which contained the most radioactivity was analyzed, except for patient J. A. in whom the first two bile samples were combined. The neutral lipid fraction contained less than 5% of the radioactivity. Recovery from the acidic steroid fraction was 92.6 (mean) ±1.9% (SD) for ^{3}H and 93 ±4.3 for ^{14}C of the total radioactivity in bile. By thin-layer chromatography 86.8 ±6.9% of the ^{3}H and 90.2 ±4.2% of the ^{14}C radioactivity in the methylated acidic steroid fraction was in bands corresponding to methyl chenodeoxycholate and methyl cholate. Radioactivity in other zones, particularly in the monohydroxy bile acid zone, was insufficient for definitive identification.

In patient M. Sc. the acidic steroid fraction after methylation and acetylation was analyzed by gas-liquid chromatography using a stream splitting technique. As shown in Fig. 1, radioactivity (^{3}H and ^{14}C) appeared in two peaks corresponding exactly in retention times to that of methyl chenodeoxycholate diacetate and methyl cholate triacetate. For each bile acid the ^{3}H:^{14}C ratio remained constant. However, it is evident that chenodeoxycholate is richer in radioactivity, especially

[2] *Abbreviations used in this paper:* GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

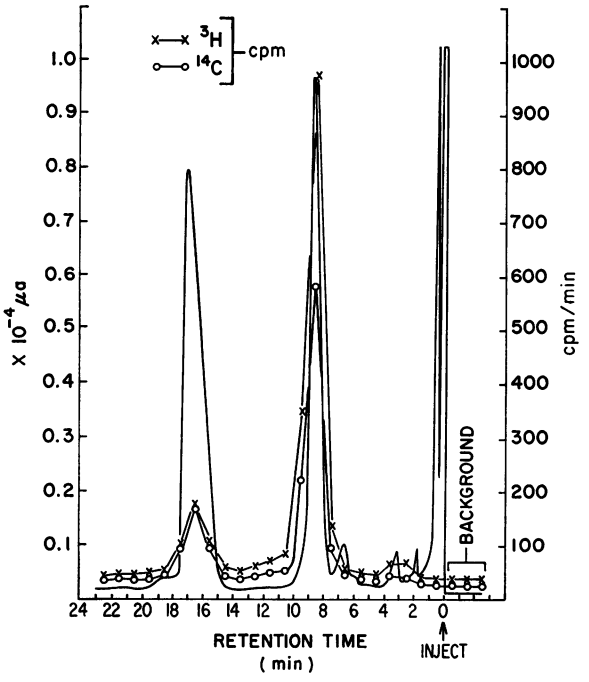

FIGURE 1 Gas-liquid chromatography of bile acid methyl ester acetates from bile of a patient (M. Sc.) given 26-hydroxycholesterol-^{3}H and 7α-hydroxycholesterol-^{14}C. Using a flame detector and stream splitting it was possible to compare the retention time of mass (uninterrupted line) and radioactivity. The peaks of radioactivity coincide with the mass responses for chenodeoxycholate and cholate (retention times 8.5 and 17.0 min respectively). The ratio of radioactivity: mass is greater for chenodeoxycholate than for cholate.

^{3}H, than cholate. This finding was confirmed by other analyses described below.

Methyl chenodeoxycholate and methyl cholate were further purified by repeated separations on thin-layer plates and/or alumina-column chromatography. Fig. 2 shows the cholic acid content and amounts of ^{14}C and ^{3}H radioactivity in fractions eluted from an alumina-column. A change in the solvent system during the elution caused corresponding changes in both mass and radioactivity. The ^{3}H: ^{14}C ratios remained constant (7.76 [mean] + 0.24 [SD]) during elution of the peak. Multiple fractions from alumina-column chromatography of methyl chenodeoxycholate from the same patient also resulted in constant ^{3}H: ^{14}C ratios of 13.18 + 0.75.

Results of crystallization to constant specific activity for M. Sc. are shown in Table II. Crystals were readily obtained after several thin-layer or column purifications, and there was little subsequent fall in radioactivity. The final products, after preparation of suitable derivatives, gave a single peak by GLC with retention times corresponding to the authentic compound. The melting points of the final crystals correspond to the standards in our laboratory and to reports in the literature (Table II). Furthermore, the ^{3}H: ^{14}C ratios of the final crystals, and also of the GLC peaks collected by stream splitting, were within one SD of the mean of the ^{3}H:^{14}C ratios of the alumina-column fractions, for both chenodeoxycholate and cholate. Thus there was no discordant loss of ^{3}H relative to ^{14}C in the various purification procedures used. These findings indicate that both hydroxycholesterols are metabolized to the same bile acids.

Relative amounts of 26-hydroxycholesterol-^{3}H and 7α-hydroxycholesterol-^{14}C metabolized to chenodeoxycholate and cholate. The per cent of each hydroxysterol metabolized to each primary bile acid was determined in the patients that received both labeled compounds (Table III). This was done in six patients by study of the distribution of ^{3}H and ^{14}C between cholate and chenodeoxycholate on the silica gel plates. The same determination was made in four patients from the specific activities of the crystalline bile acids and the relative proportion of cholate and chenodeoxycholate in bile as measured by GLC. The results of these two independent estimates were in agreement (Table III), again indicating that there is no disproportionate loss of radioactivity from either bile acid during crystallization procedures.

It is evident (Table III) that in the six patients receiving both hydroxycholesterols, a greater amount of 26-hydroxycholesterol, relative to 7α-hydroxycholesterol, was metabolized to chenodeoxycholate. Using the method of paired comparisons (13) this difference is statistically significant ($P < 0.002$).

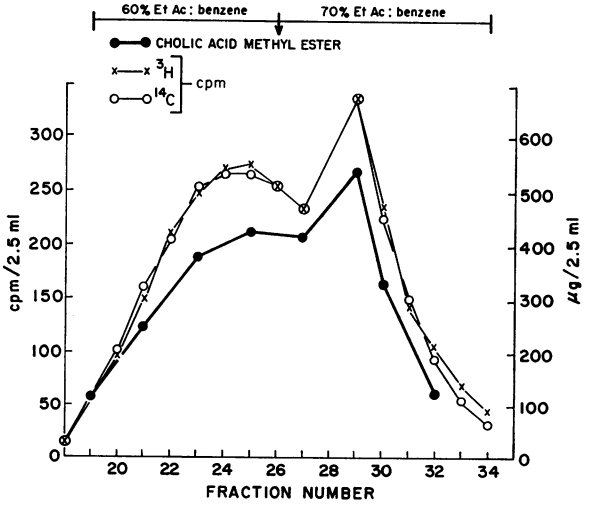

FIGURE 2 Alumina-column chromatography of purified methyl cholate (patient M. Sc.). Fractions were obtained by elution with a mixture of ethyl acetate-benzene 6:4, followed by a mixture of the same solvents 7:3. Samples were taken for quantitation of mass by GLC and radioactivity by liquid scintillation spectrometry, using internal standards for both procedures.

TABLE II

Specific Radioactivities and Melting Points of Crystalline Bile Acids Obtained from Bile (Patient M. Sc.)

Bile acids and derivatives	Radioactivity		Crystals from patient	Melting point	
	^3H	^{14}C		Authentic crystals	Literature (11, 12)
	dpm/μmole			°C	
Chenodeoxycholic acid					
Methyl chenodeoxycholate					
First crystallization	19864	1712			
Second crystallization	19529	1636			
Chenodeoxycholic acid					
First crystallization	18351	1507			
Second crystallization	19203	1400	143–144	142–144	140–146
Cholic acid					
Methyl cholate					
First crystallization	3507	445			
Second crystallization	3367	443			
Methyl cholate cathylate					
First crystallization	3518	484			
Second crystallization	3724	492	175–175.5	175–176	176–177

TABLE III

Specific Activities of Crystalline Bile Acids from Four Patients, and the Relative Amounts of Hydroxysterols Metabolized to Chenodeoxycholate and Cholate in Six Patients

Patient	Cholate in bile	Isotope	Specific activities of crystalline derivatives		Hydroxysterols metabolized to cholate	
			Chenodeoxy-cholate	Cholate	Calculated*	Found‡
	%§		*dpm/μmole*		%	
J. A.	58	^3H	15980	6666	43	38
		^{14}C	1214	1124	63	58
M. Pi.	75	^3H	6538	1844	46	48
		^{14}C	647	406	65	66
J. K.	67	^3H	2334	577	33	37
		^{14}C	491	250	51	53
M. Sa.	80	^3H	—	—	—	40
		^{14}C	—	—	—	52
M. Sc.	58	^3H	19203	3724	21	21
		^{14}C	1400	492	33	31
S. B.	77	^3H	—	—	—	59
		^{14}C	—	—	—	66

* Calculated as follows: % = (SA cholate × % cholate)/(SA chenodeoxycholate × % chenodeoxycholate + SA cholate × % cholate).

‡ Found by TLC and calculated as follows: % = (radioactivity in cholate band)/(radioactivity in chenodeoxycholate band + radioactivity in cholate band).

§ Cholate mass expressed as % of total primary bile acids in bile, as determined by GLC.

In all patients studied, the proportion of cholate present in the bile acids in bile obtained at the time of the study was greater than the proportion of either hydroxycholesterol metabolized to cholate. However, interpretation of this finding in these patients is difficult because of the recirculating pool of bile salts in individuals with partial bile fistulas.

DISCUSSION

These studies indicate that both 7α-hydroxycholesterol and 26-hydroxycholesterol are metabolized to primary bile acids in man, as was found previously in the rat (4, 14) and the hamster (4). Also, compared to 7α-hydroxycholesterol, the metabolism of 26-hydroxycholesterol to chenodeoxycholate was proportionally greater than to cholate. This is in agreement with data obtained in the rat (5) and the hamster (15) and is consistent with the hypothesis that 12α-hydroxylation is more difficult once the cholesterol side chain is oxidized (16).

The hydroxysterols we studied using tracer methods have not been definitely isolated from human liver (17), nor have their turnover rates been estimated. Therefore the relative extent to which they are intermediates in bile acid synthesis has not been fully defined.

Although 7α-hydroxycholesterol, like 7β-hydroxycholesterol, is a spontaneous air-oxidation product of cholesterol (18, 19), there is considerable evidence to suggest that it arises endogenously (20, 21). On the basis of studies in animals, the 7α-hydroxylation of cholesterol to 7α-hydroxycholesterol is accepted as the major initial step in bile acid synthesis (2), and is subject to feedback regulation (22, 23). The endogenous origin of 26-hydroxycholesterol seems likely as well, because it is not found in significant amounts as an air oxidation product of cholesterol (3, 17, 24) and can be synthesized by liver homogenate in vitro from cholesterol (3, 25). Its presence in human aorta (17, 26–28) and as the sulfate in the meconium (29) and feces (30) of newborn infants infers endogenous synthesis in man.

The possible role of initial side chain oxidation of cholesterol to 26-hydroxycholesterol in liver disease has provided the impetus for exploring its metabolism. In these studies in normal man, the principal metabolites are the dihydroxy and trihydroxy bile acids chenodeoxycholate and cholate. In addition, some radioactivity, which we were unable to definitely identify for technical reasons, was found in chromatographic fractions corresponding to monohydroxy bile acids. In both the bile fistula rat and the hamster, it has been possible to identify one monohydroxy bile acid, 3β-hydroxy-5-cholenoate, as a minor metabolite of 26-hydroxycholesterol (4, 5), and also to show that it produces a reduction in bile flow in these animals (31) in a manner similar to lithocholate. The recent finding of relatively large amounts of 3β-hydroxy-5-cholenoate in the urine of infants with biliary atresia (32) suggests the possibility that under abnormal circumstances initial side-chain oxidation of cholesterol may give rise to increased amounts of monohydroxy bile acids.

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