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Bile Acid Synthesis

METABOLISM OF 3β-HYDROXY-5-CHOLENOIC ACID TO CHENODEOXYCHOLIC ACID*

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Metabolism of 3β-hydroxy-5-cholenoic acid to chenodeoxycholic acid has been found to occur in rabbits and humans, species that cannot 7α-hydroxylate lithocholic acid. This novel pathway for chenodeoxycholic acid synthesis from 3β-hydroxy-5-cholenoic acid led to a reinvestigation of the pathway for chenodeoxycholic acid from 3β-hydroxy-5-cholenoic acid in the hamster. Simultaneous infusion of equimolar [1,2-^{3}H]lithocholic acid and 3β-hydroxy-5-[^{14}C]cholenoic acid indicated that the ^{14}C enrichment of chenodeoxycholic acid was much greater than that of lithocholic acid. Thus, in all these species, a novel 7α-hydroxylation pathway exists that prevents the deleterious biologic effects of 3β-hydroxy-5-cholenoic acid.

Considerable circumstantial evidence has now accumulated to indicate that monohydroxy bile acids such as lithocholic acid (3α-hydroxy-5β-cholanoic acid) and 3β-hydroxy-5-cholenoic acid are of endogenous origin (1–3) and are found in biologic fluids beginning in fetal life (4–6). Because in the adult the major source of lithocholic acid is known to be from bacterial dehydroxylation of chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid), recognition that it can also be synthesized in the liver was not appreciated. However, lithocholic acid has been found to occur in the newborn period independent of intestinal bacterial activity (7) which is consonant with the knowledge that it is derived from 3β-hydroxy-5-cholenoic acid *in vivo* in the hamster (8) and the knowledge that in humans the latter bile acid has been shown to be derived from radioactive cholesterol (5-cholesten-3β-ol) given parenterally (1, 2).

More recent studies also indicate that esterification of these monohydroxy bile acids with sulfate or the formation of glucuronides does not eliminate their deleterious biologic effects (9, 10), and therefore hydroxylation to chenodeoxycholic acid is essential.

Although we previously thought that metabolism to chenodeoxycholic acid was via lithocholic acid (11), our current findings in humans and rabbit indicate that a different pathway must exist in these species. Moreover, by using radioactive lithocholic acid and 3β-hydroxy-5-cholenoic acid infused simultaneously in the hamster, we were able to demonstrate that the major pathway for metabolism to chenodeoxycholic acid is via a pathway that by-passes lithocholic acid.

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EXPERIMENTAL PROCEDURES

Synthesis of 3β-hydroxy-5-[1,2-^{3}H]cholenoic acid followed established procedures and was given in detail in our previous paper (8). The tritiated compound used in this study is from the same synthesis as that used previously (0.9 mCi/μmol); but when supplies were exhausted, it was decided to prepare the isotopic compound labeled at C-24 with ^{14}C because of the theoretical possibility that loss of tritium could occur from C-2 during the formation of the 4-ene-3-one intermediate that occurs in the synthesis of lithocholic and chenodeoxycholic acids. Also, unlike the previous synthesis in which isomers occur during sodium borohydride reduction, no alteration of the A ring is necessary, thus simplifying the purification procedure.

Synthesis of 3β-hydroxy-5-[24-^{14}C]cholenoic acid began with the preparation of norcholenyl iodide by photochemical decarboxylation (13) of the 3-acetate of the free acid. Using prepurified argon to maintain an oxygen-free atmosphere, a solution of iodine (0.533 g, 2.1 mmol) in benzene (15 ml) was added dropwise to potassium *t*-butoxide (0.157 g, 1.4 mmol) suspended in benzene (11 ml) in a foil-wrapped flask. The mixture was stirred at room temperature in the dark for 30 min and then filtered rapidly under argon through a sintered glass funnel into a solution of the 3-acetate of cholenoic acid (0.208 g, 5 mmol) in 25 ml of benzene. The solution was irradiated with a 1000-watt tungsten lamp with stirring under argon for 45 min. Sodium thiosulfate (0.1 N) was then added dropwise to discharge the purple iodine color, and the resultant yellow mixture was then extracted into benzene which was washed with water and then poured over anhydrous $MgSO_4$ and concentrated by flash evaporation. The crude product was purified by flash chromatography on silica gel (hexane:ether (10:1, v/v)) to yield norcholenyl iodide as a white solid (165 mg, 66%, m.p. 135–140 °C with decomposition). For ^{1}H NMR analysis (250 mHz, $CDCl_3$): δ 5.51 (d, J = 5 Hz, 1H, H_6), 4.60 (m, 1H, H_3), 3.31 (m, 1H, H_{23a}), 3.10 (m, 1H, H_{23b}), 2.03 (s, 3H, OAc), 1.02 (s, 3H, H_{19}), 0.93 (d, J = 6 Hz, 3H, H_{21}), 0.70 (s, 3H, H_{18}).

Preparation of the 24-^{14}C-labeled acid began by substitution of the iodide by ^{14}CN (14) as the potassium salt (specific activity = 50 mCi/mmol) (ICN Radiochemicals, Irvine, CA) which was added (0.038 mmol) to a suspension of norcholenyl iodide (24 mg, 0.048 mmol) in 1 ml of anhydrous dimethyl sulfoxide which was stirred for 5.5 h at 95–100 °C after which the nitrile derivative was obtained (19 mg) and purified by thin-layer chromatography and then converted to the carboxylate by addition of a 20 molar excess of powdered sodium hydroxide (14). The aqueous phase was then acidified to pH 2.0 with HCl, and the free acid was extracted into ether. The crude product was purified by preparative TLC as described above but with a solvent system of 1% formic acid in hexane:ether (1:1, v/v) (two developments, R_f = 0.30). The white solid that was obtained (5 mg, 53%) was analyzed by HPLC[1] as described previously and was found to be identical to our previously prepared tritiated compound. In addition, reverse isotope dilution using authentic compound (Calbiochem-

[1] The abbreviation used is: HPLC, high pressure liquid chromatography.

Behring) showed no loss of radioactivity from the calculated specific activity.

The steps used in identification of metabolites were (*a*) solvolysis, (*b*) hydrolysis, (*c*) extraction, (*d*) methylation and thin-layer chromatography, (*e*) column chromatography using Glycophase G on Controlled-Pore Glass 80–100 mesh (Pierce Chemical Co.), (*f*) HPLC using μPorasil (10-μM silica, Waters Associates) and reverse isotope dilution. All these steps have been described in detail in previous publications (8, 11) including the quantitation of radioactivity by liquid scintillation spectrometry using a Beckman CPM 200 instrument. In dual isotope studies, narrow windows were used so that less than 1% of the ^{3}H was detected in the ^{14}C channel and crossover of ^{14}C into the tritium window was 10%. The amount of each metabolite in bile was determined from the proportional distribution of radioactivity after thin-layer chromatography and confirmed by HPLC using μPorasil as described above. It was found that complete separation of lithocholic acid from 3β-hydroxy-5-cholenoic acid and of chenodeoxycholic acid from deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid) could be obtained using 20 × 20-cm plates coated with Silica Gel G and a solvent system of chloroform:acetone (9:1, v/v) followed after drying at room temperature by isooctane:isopropyl ether:glacial acetic acid (2:2:1, v/v).

Radioactive lithocholic, deoxycholic, and cholic acids were purchased from Amersham/Searle and/or New England Nuclear. Radioactive 3-keto-5β-cholanoate and 3α-hydroxy-5β-cholanoate were prepared from lithocholic acid by chromate oxidation and sodium borohydride reduction. These compounds were purified by thin-layer and column chromatography and used as standards.

For intravenous administration of tracer amounts of radioactive bile acids, the compounds were dissolved in 0.05 ml of absolute ethanol and added with vortexing to 5 ml of sterile 5% dextrose in 0.9% NaCl containing 5% human serum albumin. Studies in hamsters required larger amounts of bile acids; and for this purpose, the $^{3}H/^{14}C$ isotope ratio of lithocholic and 3β-hydroxy-5-cholenoic acids was adjusted to provide an approximate 3:1 counting ratio. Then, nonradioactive bile acids were added to provide a final total concentration of 900 nmol/ml. This mixture was prepared initially in methanol and then taken to dryness using vacuum. The compounds were then redissolved with heating to 60 °C in 0.2 ml of propylene glycol to which 0.8 ml of the sterile solution containing albumin as described above was added.

Two individuals that required T-tube drainage following cholecystectomy for gallstones consented to the intravenous administration of tracer amounts of bile acids.

Male and female New Zealand rabbits weighing between 1.5 and 4 kg received intravenous pentobarbital anesthesia, which was maintained throughout the study. The abdomen was opened by a midline incision, and a PE 90 polyethylene cannula was inserted into the common duct and brought out through the incision, which was then closed. Bile was drained into graduated tubes for the determination of volume.

Male Syrian hamsters weighing from 100 to 112 g were used and prepared as described previously in detail (8, 11). Infusions of equimolar amounts of lithocholic and 3β-hydroxy-5-cholenoic acids were given immediately after surgery before depletion of the endogenous bile acid pool and changes in the basal rate of bile acid synthesis.

RESULTS

Table I summarizes the studies in humans, rabbits, and hamsters. Recovery of radioactivity was virtually complete except in the human studies with partial biliary drainage. In these studies, recovery of simultaneously administered [24-^{14}C]chenodeoxycholic acid was greater than that of administered 3β-hydroxy-5-[1,2-^{3}H]cholenoic acid, and this discrepancy could not be accounted for by analysis of the urine collected during the same time period.

In rabbits, HPLC analysis of the radioactive metabolites indicated the presence of two bile acids in addition to lithocholic acid and chenodeoxycholic acids (Fig. 1). One metabolite, Peak A, was less polar than lithocholic acid; and the relative retention time did not change following an acetylation procedure, suggesting the presence of a 3-oxo compound. However, the retention time did not correspond to a standard of 3-oxo-5β-cholanoic acid. The second unidentified metabolite, Peak C, was slightly less polar than chenodeoxycholic acid, formed an acetate, but was not identical in retention time to ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid). In two studies, the unidentified metabolites accounted for 31 and 40% of the total metabolites. Final identification of chenodeoxycholic acid was by reverse isotope dilution from an eluate after HPLC chromatography (Table II).

The distribution of the bile acids in hamster bile derived

TABLE I

Distribution of radioactivity recovered in bile following intravenous administration of bile acids

Study	Bile acid	μCi	Recovery	Time	Distribution			
					3-OH[a]	Litho	Cheno	Cholic
			%	*h*	%			
Patients								
1A	[^{3}H]3β-OH	10	30	24	70	4	26	0
1B	[^{3}H]3β-OH	10	34	24	83	3	14	0
	[^{14}C]Cheno	10	90	24	0	0	100	0
2	[^{3}H]3β-OH	10	30	24	92	0	8	0
	[^{14}C]Cheno	10	72	24	0	0	100	0
Rabbits								
3[b]	[^{3}H]3β-OH	11	100	5.5	36	29	4	0
	[^{14}C]Litho	1.9	100	5.5	0	100	0	0
6	[^{3}H]3β-OH	57	84	7.7	ND	ND	4	0
	[^{14}C]Litho	4.9	65	7.7	0	100	0	0
13	[^{14}C]3β-OH	3.2	83	2.0	14	40	6	0
Hamsters								
1	[^{14}C]3β-OH	0.13	100	18	67	5	28	0
	[^{3}H]Litho	0.5	100	18	0	36	64	0
2	[^{14}C]3β-OH	0.13	97	18	55	10	35	0
	[^{3}H]Litho	0.5	86	18	0	37	62	0
3	[^{14}C]3β-OH	0.13	100	18	65	6	29	0
	[^{3}H]Litho	0.5	100	18	0	36	64	0

[a] 3-OH, 3β-hydroxy-5-cholenoic acid; [^{3}H]3β-OH, 3β-hydroxy-5-[1,2-^{3}H]cholenoic acid; [^{14}C]cheno, 3α,7α-dihydroxy-5β-[24-^{14}C]cholanoic acid; [^{14}C]litho, 3α-hydroxy-5β-[24-^{14}C]cholanoic acid; [^{14}C]3β-OH, 3β-hydroxy-5-[24-^{14}C]cholenoic acid; ND, not analyzed by HPLC.

[b] In rabbits, two radioactive peaks were detected in addition to lithocholic and chenodeoxycholic acids.

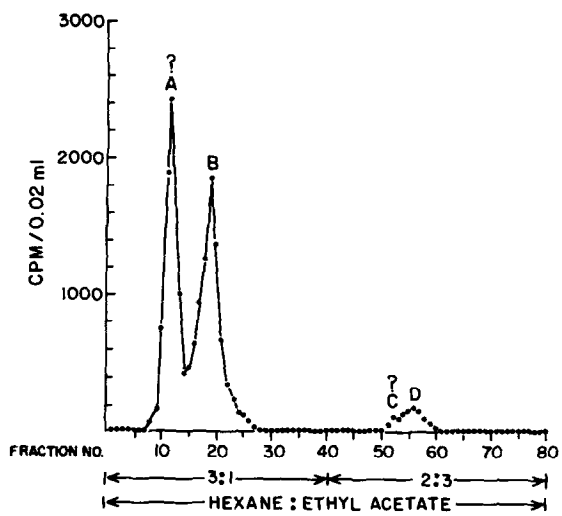

FIG. 1. **Glycophase analysis of metabolites of 3β-hydroxy-5-cholenic acid in rabbit bile as methyl esters.** *Peaks B* and *D* were identified as lithocholic acid and chenodeoxycholic acid, respectively, by HPLC analysis as the methyl ester acetates and by reverse isotope dilution. *Peak A* was unchanged in retention time after an acetylation procedure but did not have a retention time identical to 3-oxo-5β-cholanoic acid. *Peak C* did shift in retention time after acetylation but was not identical to ursodeoxycholic acid.

from the administered 450 nmol of lithocholic and 3β-hydroxy-5-cholenoic acids of known specific activities is shown in Table III. The amount of 3β-hydroxy-5-cholenoic acid metabolized to lithocholic acid in each study is much less than the amount metabolized to chenodeoxycholic acid. As shown, determination of the fraction of newly synthesized chenodeoxycholic acid derived from 3β-hydroxy-5-cholenoic acid via metabolism to lithocholic acid can be estimated from its proportion in the lithocholic acid in bile and the amount of lithocholic acid that was also metabolized to chenodeoxycholic acid. In each study, less than 40% of the total chenodeoxycholic acid derived from 3β-hydroxy-5-cholenoic acid could have been derived via lithocholic acid as an intermediate.

TABLE II

Identification of chenodeoxycholic acid as a metabolite of 3β-hydroxy-5-cholenoic acid by reverse isotope dilution

Study	Chenodeoxycholic acid diacetate	
	$^3H/^{14}C$	Ratios
	cpm	
Patient 1A		
1st crystallization[a]	2118:339[b]	6.25
2nd crystallization	2180:348	6.26
Rabbit 13		
1st crystallization	149:85[b]	1.75
2nd crystallization	131:74	1.78
3rd crystallization	144:81	1.78

[a] Successive crystallizations from methanol.

[b] To the methyl ester eluate from the HPLC column, a standard of either [^{14}C]- or [^{3}H]chenodeoxycholic acid methyl ester was added and then the diacetate was prepared. After HPLC as a diacetate, an eluate was taken for reverse isotope dilution using 50 mg of authentic chenodeoxycholic acid methyl ester diacetate.

TABLE III

Metabolism of 3β-hydroxy-5-cholenoic acid and lithocholic acid in the hamster following simultaneous administration of equimolar amounts

An equimolar mixture of [^{3}H]lithocholic acid and 3β-hydroxy-5-[^{14}C]cholenoic acid was prepared as described under "Experimental Procedures," and 0.5 ml containing 450 nmol of each bile acid was injected intravenously into a bile fistula animal over a period of 1–2 min. Maximum estimate of the number of nanomoles of chenodeoxycholic derived from 3β-hydroxy-5-cholenoic acid via lithocholic acid as an intermediate is based on the $^{14}C/^3H$ ratio found in bile. See Table I for abbreviation definitions.

Hamster	3β-OH	Litho	Cheno	[^{14}C]Litho[a]	[^{14}C]Cheno via litho[b]
	nmol	*nmol*	*nmol*	*nmol %*	*nmol*
1					
3β-OH	302	23	125	12	39
Litho	0	162	288		
2					
3β-OH	240	34	163	20	62
Litho	0	139	248		
3					
3β-OH	293	27	130	14	47
Litho	0	162	288		

[a] nmol of [^{14}C]litho/(nmol of [^{3}H]litho + nmol of [^{14}C]litho) × 100.

[b] nmol % [^{14}C]litho × nmol of [^{3}H]cheno/(100 − nmol % [^{14}C] litho).

DISCUSSION

The experimental model for the induction of cholestasis using either lithocholic acid or 3β-hydroxy-5-cholenoic acid (18) was confirmed in a number of laboratories (9, 10) and was followed by the identification of these monohydroxy bile acids in normal human amniotic fluid (6) and meconium (5) and was found to represent as much as one-third of the total bile acids in the urine of infants with biliary atresia (1). Administration of radioactive cholesterol to one of these infants (1) was followed by the excretion of radioactive 3β-hydroxy-5-cholenoic acid. In this study, the isotopic enrichment of 3β-hydroxy-5-cholenoic acid was less and was more prolonged than that found for chenodeoxycholic or cholic acids (3α,7α,12α-trihydroxy-5β-cholanoic acid), implying either a different precursor-product relationship or distribution *in vivo* in a relatively large pool that turns over slowly. Our present findings also imply a different distribution of 3β-hydroxy-5-cholenoic acid in humans.

Although esterification of monohydroxy bile acids with sulfate or the formation of glucuronides can reduce or eliminate the deleterious effects on bile flow (9), considerable evidence now exists to indicate that many of these derivatives, such as lithocholic acid glucuronide (10), can also induce cholestasis. In contrast, chenodeoxycholic acid and its conjugates and sulfate ester (12) when administered intravenously cause an increase in bile flow. From these findings, it is reasonable to conclude that the major pathway for prevention of the deleterious biologic effects is 7α-hydroxylation, which after further enzymatically catalyzed transformations yields chenodeoxycholic acid.

Further investigation of the pathway for chenodeoxycholic acid synthesis in the hamster brings into harmony the observations in humans and rabbits. All these species metabolize 3β-hydroxy-5-cholenoic acid to chenodeoxycholic acid by a pathway that does not have lithocholic acid as an intermediate. In these studies, the amount of 3β-hydroxy-5-cholenoic acid found in bile that was metabolized to lithocholic acid is

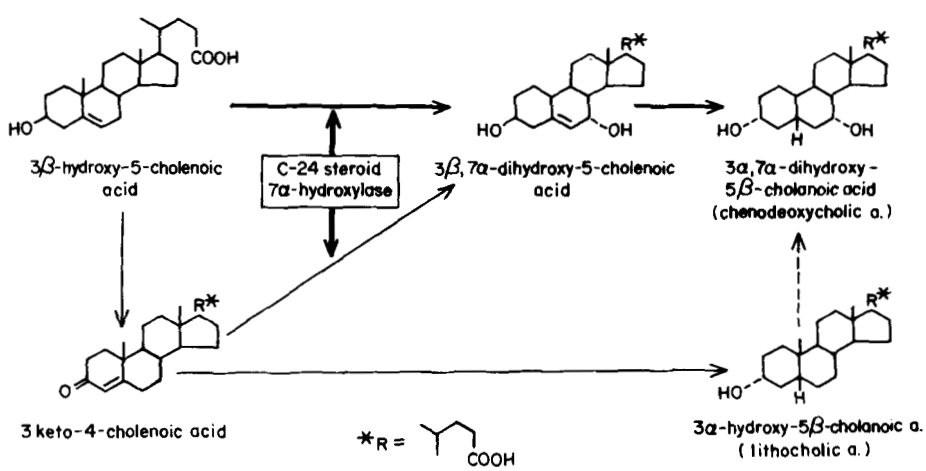

FIG. 2. **Metabolic pathway(s) for the synthesis of chenodeoxycholic acid from 3β-hydroxy-5-cholenoic acid.** In the three species studied, a metabolic pathway that by-passes lithocholic acid as an intermediate has been identified. Although 3-oxo-4-cholenoic acid when given intravenously is metabolized to chenodeoxycholic acid, it is not certain which is the preferred substrate for 7α-hydroxylation in the endogenous pathway. In addition, in the hamster, as illustrated by the *interrupted arrow*, a pathway exists for the metabolism of lithocholic acid to chenodeoxycholic acid.

less than the amount metabolized to chenodeoxycholic acid. Since the newly synthesized [^{14}C]lithocholic acid was distributed in the liver with the [^{3}H]lithocholic acid that was given simultaneously, the fraction of 3β-hydroxy-5-cholenoic acid metabolized to chenodeoxycholic acid via this pathway should be proportional to the total amount of lithocholic acid metabolized to chenodeoxycholic acid. Using the proportion of 3β-hydroxy-5-cholenoic acid metabolized to lithocholic acid found in bile as an estimate of the proportion in liver probably overestimates this ratio since enrichment of the lithocholic acid pool with [^{14}C]lithocholic acid was negligible immediately following injection and increased with time. Thus, the small fraction of chenodeoxycholic acid derived via lithocholic acid from 3β-hydroxy-5-cholenoic is probably an overestimate, and the major pathway is via a different pathway as illustrated in Fig. 2.

The identification of relatively small amounts of 3β,7α-dihydroxy-5-cholenoic acid in bile (15) together with the knowledge that it is rapidly metabolized to chenodeoxycholic acid after intravenous infusion (16) supports the view that it is a naturally occurring intermediate in bile acid synthesis. Although the present studies indicate that this allylic bile acid can be derived by 7α-hydroxylation of 3β-hydroxy-5-cholenoic acid, they do not exclude the possibility that in the natural pathway addition of the 7α-hydroxyl group occurs prior to complete oxidation of the side chain of cholesterol to the C-24 bile acid.

The amount of monohydroxy bile acid formed endogenously is dependent on the activity of one or more 7α-hydroxylases. From the knowledge gained previously in regard to hydroxylation of bile acids (17), it is likely that the proposed steroid 7α-hydroxylase(s) will prove to be among the microsomal P-450 enzymes. The level of their enzymatic activity may be a critical determinant of the proportion of monohydroxy bile acid found in the newborn period.

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