

Synthesis of 11,12-²H₂- and 11,12-³H₂-labeled chenodeoxycholic and lithocholic acids

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Abstract Deuterium- and tritium-labeled chenodeoxycholic acid and lithocholic acid were prepared by catalytic reduction of their respective Δ^{11} derivatives. Structures of the intermediates and their isotopic purity were verified by chemical ionization and electron impact mass spectrometry and by nuclear magnetic resonance spectroscopy. Experimental conditions for reductive deuteration were defined which gave complete reduction of the olefin and a product of high isotopic purity. Conditions for optimal tritiation were developed with which little exchange of protons with the solvent occurred; the product had high specific activity. To test biological stability of the label, the ³H-labeled chenodeoxycholic acid was administered simultaneously with ¹⁴C-labeled chenodeoxycholic acid to two healthy subjects and the ³H/¹⁴C ratio in bile was determined daily for several days. The ratio remained identical to that administered, suggesting that the 11,12-³H label in chenodeoxycholic acid is stable during enterohepatic cycling and can be used for valid estimates of bile acid kinetics in man by the isotope dilution technique.

Supplementary key words 3 α -hydroxy-5 β -[11,12-³H]cholanolic acid · 3 α ,7 α -dihydroxy-5 β -[11,12-³H]cholanolic acid · deuterium labeling · chemical ionization mass spectrometry · cholonic acids · stable isotopes

The preparations of tritium- or deuterium-labeled bile acids presently available for clinical investigation suffer from a number of limitations. Bile acids have been labeled with ³H at the 2 and 4 positions by enolic exchange of the 3-keto derivative on basic alumina containing tritiated water (1), but the product has a low specific activity and the label is partially removed by the enteric flora in man (2–4). Cholic acid labeled with ³H in the 7 β position has been prepared by [³H]borohydride reduction of the 7-keto derivative and used for studies of bile acid metabolism in man (5), but this label will be lost if bacterial dehydrogenation occurs at the 7 position. Randomly labeled bile acids of high specific activity may be prepared by Wilzbach exchange (6); but the site of labeling is unknown, and the biologic stability of one such preparation has been unsatisfactory (4) and another imperfect (7). Deuterium-labeled bile acids are particularly useful for studies in pregnancy and during childhood (8); although 2,4-²H-labeled bile

acids can be prepared by enolic exchange, they are difficult to obtain with high isotopic purity (9).

We report here a method for the preparation of deuterium- and tritium-labeled chenodeoxycholic and lithocholic acids by heterogeneous catalytic reduction of the Δ^{11} precursors. Conditions for optimal incorporation of label have been examined. The stability in vivo of the label in [11,12-³H]chenodeoxycholic acid was characterized in preliminary experiments in two healthy subjects.

METHODS

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian HA-100 NMR spectrometer (Varian Associates, Palo Alto, CA), and chemical shift data are presented in parts per million (ppm) relative to tetramethylsilane as an internal standard.

Mass spectrometry

Electron impact (EI) mass spectra were recorded at Argonne National Laboratory using a Perkin-Elmer model 270 mass spectrometer (Perkin-Elmer Corp., Norwalk, Conn.) with an ionization voltage of 70 eV at a source temperature of 150°C. EI spectra obtained at Syntex Research (Palo Alto, CA) were recorded on a Varian MAT CH-7, also at 70 eV ionization voltage. Chemical ionization (CI) mass spectra were obtained at Argonne National Laboratory using a Biospect (Scientific Research Instruments, Baltimore, MD) CI

Abbreviations: GLC, Gas-liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; EI, electron impact; CI, chemical ionization.

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mass spectrometer at a source pressure of 0.8 mm of isobutane or methane and a source temperature of 150°C.⁶

Both CI and EI mass spectrometry were used in the analysis of the deuterium-labeled bile acid products. The ions from the olefin and the deuterated product are separated by four mass units and, if it is assumed that both species will exhibit the same or very similar ionization properties, the ion intensities can be used to determine the mole composition of the saturated product.

Isotope purity

Isotopic composition was calculated by correcting the observed ion intensities for the small proportion of olefin containing two ¹³C atoms at natural abundance which will contribute to the apparent content of dideuterated bile acid. Deuterium gas (99.5 atom % ²H) was obtained from Matheson Gas Products, Lyndhurst, NJ 07071. Deuterated solvents, [²H₆]acetone, [²O²H]ethanol and [²H₄]acetic acid (99.5 atom % ²H), were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI 53233.

Radiochemical purity

[24-¹⁴C]Chenodeoxycholic acid (Mallinckrodt, St. Louis, MO), previously shown by zonal scanning to be >98% radiopure (after crystallization four times from ethyl acetate), and [11,12-³H₂]chenodeoxycholic acid were cocrystallized several times from ethyl acetate, and samples of the crystals were counted to determine ³H/¹⁴C ratios. Recrystallization from methanol-water was performed in a similar manner for [24-¹⁴C]lithocholic acid (supplied by Dr. Robert H. Palmer; >98% radiopure by zonal scanning after one purification by preparative TLC) and [11,12-³H₂]lithocholic

acid. Radioactivity was determined by scintillation counting.

Chromatography of products

Resolution of saturated and unsaturated bile acid methyl ester acetates was achieved by argentation thin-layer chromatography (TLC) (10) on glass plates coated to a thickness of 250 μm with a slurry of silica gel H containing aqueous silver nitrate (12.5%, wt/vol). The TLC plates were developed with benzene-ether 80:20 (v/v). The bile acid methyl esters could not be separated by TLC unless the hydroxyl groups were acetylated.

Gas-liquid chromatography (GLC) of methyl ester acetates gave less satisfactory resolution of the saturated and unsaturated bile acids, but partial resolution could be achieved at 235°C with a 6-ft (2 mm ID) column packed with 1% SP-1000 (Supelco, Inc., Bellefonte, PA).

Melting points were determined on a Fisher-Johns melting point apparatus and are reported uncorrected.

Preparation of bile acid olefins

Unsaturated bile acids were prepared by a modification of a procedure developed by Nakada and Yamasaki (11,12). Fig. 1 shows the preparation of [11,12-²H₂]chenodeoxycholic acid.

3α-Hydroxy-Δ¹¹-5β-cholenic acid

Methyl 3α-acetoxy-12α-hydroxy-5β-cholanate (5.0 g, 11.2 mmole), prepared by partial acetylation of methyl deoxycholate (13), was dissolved in 40 ml of pyridine. Fifteen ml of phosphorus oxychloride was added and the solution was maintained at 55°C for 24 hr. The reaction was quenched in ice water, and the product was extracted into ether. The crude product was crystallized three times from aqueous methanol, mp 117–119°C (reported [11] mp 116–118°C). TLC on silver nitrate-impregnated silica gel plates and development with benzene-ether 80:20 (v/v), gave *R_f* values of 0.78 for methyl 3α-acetoxy-5β-cholanate (under these conditions methyl 3α-acetoxy-Δ¹¹-5β-cholenate had an *R_f* value of 0.44). The NMR spectrum of the product showed two characteristic vinyl proton resonances at 6.10 ppm (1H, dd, *J* = 11 and 3 Hz) and 5.40 ppm (1H, dd, *J* = 11 and 1.5 Hz). The CI mass spectrum showed ions at *m/e* 431 [MH⁺] and *m/e* 371 [MH⁺-HOAc]. The EI spectrum showed only a very weak molecular ion at *m/e* 430 and more intense characteristic ions at *m/e* 370 [M⁺-HOAc] and *m/e* 255 [M⁺-HOAc⁻side chain]. The ester was saponified by brief refluxing in 5% alcoholic KOH solution. The solution was

⁶ A report of the chemical ionization mass spectra of bile acids will be presented elsewhere by D. L. Hachey and P. D. Klein.

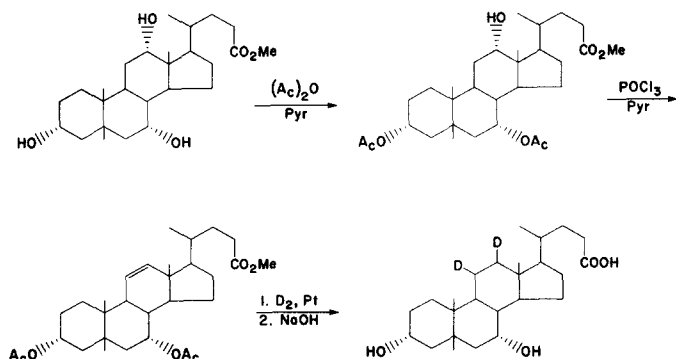


Fig. 1. Synthesis of [11,12-²H₂]chenodeoxycholic acid from cholic acid.

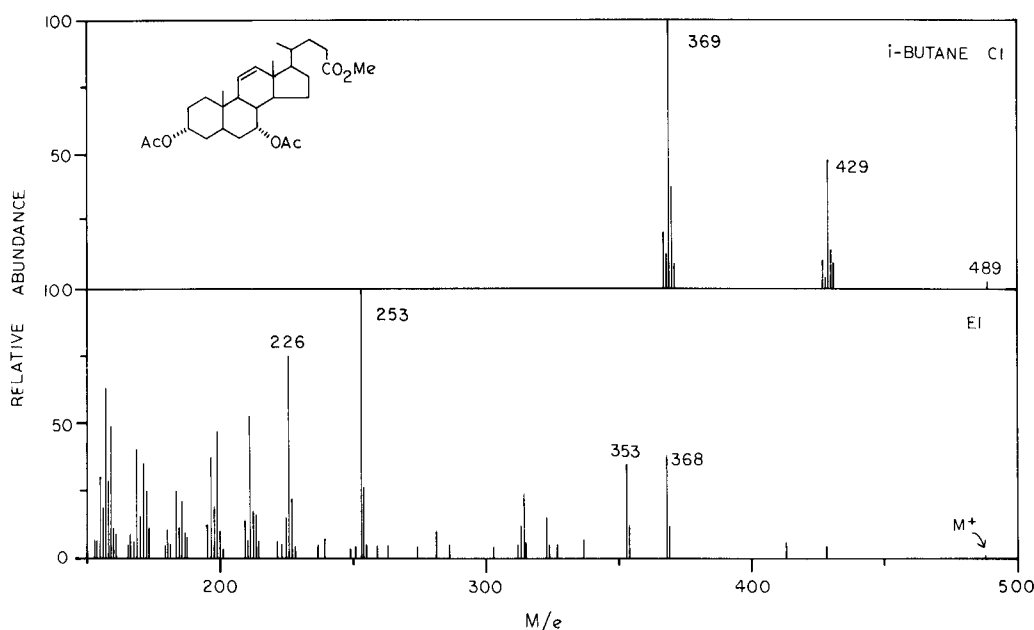


Fig. 2. Chemical ionization (top) and electron impact (bottom) mass spectra of methyl $3\alpha,7\alpha$ -diacetoxy- Δ^{11} - 5β -cholenate.

acidified and the product was extracted into ether, which was dried and concentrated. The product precipitated from ether as an amorphous solid, mp 165–167°C (reported [12] mp 168–169°C).

$3\alpha,7\alpha$ -Dihydroxy- Δ^{11} - 5β -cholenic acid

Methyl $3\alpha,7\alpha$ -diacetoxy- 12α -hydroxy- 5β -cholanate was prepared from cholic acid as described [13], and dehydrated with phosphorus oxychloride as described above. The product, methyl $3\alpha,7\alpha$ -diacetoxy- Δ^{11} - 5β -cholenate, was purified by crystallization from methanol, mp 138–140°C (reported [12] mp 139–141°C). TLC, as described above, gave an R_f value of 0.51 for methyl $3\alpha,7\alpha$ -diacetoxy- 5β -cholanate (under these conditions methyl $3\alpha,7\alpha$ -diacetoxy- Δ^{11} - 5β -cholenate had an R_f value of 0.35). The NMR spectrum of the olefin was in full agreement with the assigned structure, exhibiting two vinyl proton resonances centered at 6.15 (1H, dd, $J = 10$ and 3 Hz) and 5.45 ppm (1H, dd, $J = 10$ and 1.5 Hz) for the C-11 and C-12 hydrogens, respectively. The CI mass spectrum (Fig. 2) showed a simple spectrum with only three important ions at m/e 489 [MH^+], m/e 429 [$MH^+ - HOAc$], and m/e 369 [$MH^+ - 2HOAc$]. The EI mass spectrum showed characteristic ions at m/e 368 [$M^+ - 2HOAc$], m/e 353 [$M^+ - 2HOAc - CH_3$], and m/e 253 [$M^+ - 2HOAc - \text{side chain}$]. The product was saponified in 5% methanolic KOH to give $3\alpha,7\alpha$ -dihydroxy- Δ^{11} - 5β -cholenic acid. The product crystallized from ethyl acetate as colorless plates, mp 206–209°C (reported [12] mp 204–206°C).

Catalytic reduction of bile acid olefins

Optimal conditions for incorporation of deuterium. The goal was to find conditions giving complete reduction to a product with high isotopic purity. For convenience of analysis and purification of the product, we used the $3,7$ -diacetoxy derivative of chenodeoxycholic acid, but the results obtained should apply equally to the unprotected olefin. Conditions that were tested and the isotopic compositions of the products are summarized in Table 1. A typical deuteration using the selected conditions is outlined below.

Methyl $3\alpha,7\alpha$ -diacetoxy- 5β - Δ^{11} -cholenate, 180.3 mg (0.369 mmole), was dissolved in 5 ml of [O^2H]ethanol containing 194 ml of platinum oxide and 1 ml of [2H_4]acetic acid as catalyst. The reduction was run at room temperature under deuterium gas (10 lb/in²) for 15 min. By CI (Fig. 3), the product contained 98.3% saturated product with high isotopic purity (3% 2H_0 , 16% 2H_1 , and 81% 2H_2). Protecting groups were removed by saponification induced by addition of KOH (2 g/5 ml water) for 2 days at 25°C. The ethanolic solution was acidified with 1 N HCl and the acid was extracted with ether. TLC analysis (benzene–dioxane–acetic acid 55:40:2) showed a mixture of two products in a 4:1 ratio (R_f 0.43). The major product had the mobility of chenodeoxycholic acid. The minor product was presumably the 7-acetate. The product was purified by column chromatography on silica gel using a gradient of methanol in chloroform. Fractions eluting with 7–15% methanol contained [$11,12\text{-}^2H_2$]chenodeoxycholic acid.

TABLE 1. Effect of conditions on reduction of the 3,7-diacetoxy- Δ^{11} olefin and isotopic composition of saturated product

Experiment ^a	Olefin <i>mmoles</i>	Solvent <i>ml</i>	PtO ₂ Catalyst <i>mg</i>	P atm <i>lb/in²</i>	Time <i>hr</i>	Reduction <i>%^b</i>	Saturated Product ^c		
							² H ₀	² H ₁	² H ₂
1	0.15	² H ₆ -acetone, 5	100	10	42	71	23	44	33
2	0.10	² H ₆ -acetone, 5	100	10	20	98	23	46	31
3	0.67	O ² H-ethanol, 10	126	18	1	67	6	13	81
3a	0.67	O ² H-ethanol, 10	202	18	1	52 (84)	5	14	81
4	0.37	O ² H-ethanol, 5 ² H ₄ -acetic acid, 1	194	10	0.25	98	4	16	80

^a All reductions were carried out at room temperature. In experiment 1, prerduced catalyst was used, whereas in all other experiments the catalyst was reduced in situ. In experiment 3a, the material used was the product of experiment 3, i.e., it contained only 33% olefin, and at the completion of the reduction, 84% of the material has been reduced. The product of reaction 3a contained three products (resulting from loss of acetyl groups) when examined by thin-layer chromatography.

^b Percentage reduction was estimated by CI mass spectrometry. See text.

^c Percentage of deuterated species was calculated from the ion intensities of the saturated products by mass spectrometry. The data are corrected for the ¹³C fragments originating from the natural abundance of ¹³C in adjacent masses.

Earlier fractions contained the impurity. Fractions containing chenodeoxycholic acid were combined and evaporated to dryness. The product was dissolved in a small volume of ethyl acetate and treated with hexane just to the point of turbidity, at which point crystallization began. The yield was 51.6 mg (0.131 mmole, 36%) of the free acid, mp 133–138°C (reported [14] mp 138°C).

Optimal conditions for incorporation of tritium. The goal was to find conditions yielding a product of high specific activity. Exchange of protons with solvent had to be minimized; complete reduction of the olefin was deemed less important. Optimal conditions for incorporation of tritium were defined using deu-

terium for convenience. We elected to use the unprotected olefin to facilitate purification of the final, highly radioactive product which would be obtained after tritiation. Results are given for representative reductions with deuterium gas in **Table 2**. A typical tritiation using the selected conditions is described here. 3 α -Hydroxy- Δ^{11} -5 β -cholenic acid (50 mg) or 3 α ,7 α -dihydroxy- Δ^{11} -cholenic acid (50 mg) was dissolved in 2 ml of dioxane containing 50 mg of platinum oxide. The double bond was reduced with tritium gas for 30 min.⁷ Hydrogen gas was added to complete

⁷ Tritiation of olefinic bile acids was carried out by New England Nuclear, Boston, Massachusetts, using carrier free tritium gas and conditions specified in the text.

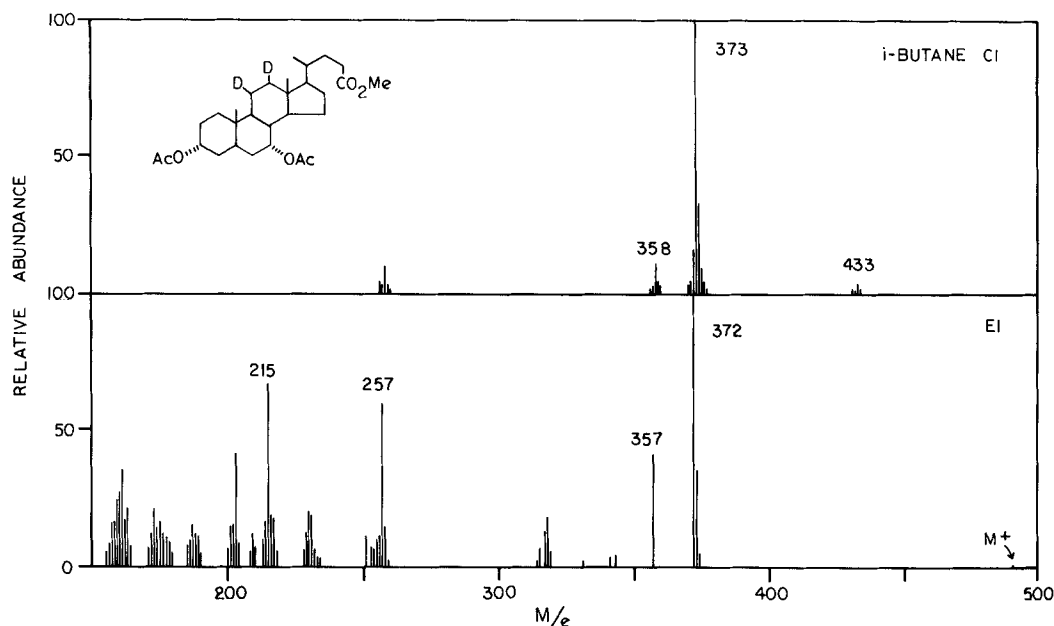


Fig. 3. Chemical ionization (top) and electron impact (bottom) mass spectra of methyl 3 α ,7 α -diacetoxy-5 β -[11,12-²H]cholanate.

TABLE 2. Effect of conditions on reduction with deuterium of the 3 α -hydroxy- Δ^{11} olefin and isotopic composition of the product

Solvent	Catalyst ^a	% Reduction ^b	% ² H ₀ ^c	% ² H ₁ ^c	% ² H ₂ ^c
Acetic acid	PtO ₂	68	60	18	22
Isopropanol	PtO ₂	83	76	11	13
Isopropanol	5% Pd-C	94	16	35	49
Dioxane	PtO ₂	62	20	20	60

^a Reduction conditions as in experiment 3, Table 1.

^b Percentage reduction was estimated by CI mass spectrometry. See text for discussion.

^c Percentage of deuterated species was calculated from the ion intensities of the saturated products by mass spectrometry. The data are corrected for the natural abundance of ¹³C contributed by ions at adjacent masses.

the reduction. Labile tritium was removed under vacuum with ethanol as the solvent. The tritiated bile acids were stored in ethanol. The specific activity, assuming complete reduction of the olefin, was 1.5 Ci/mmmole.

Biologic Stability

[11,12-³H₂]Chenodeoxycholic acid and [11,12-³H₂]lithocholic acid were incubated at pH 7.5 with fresh human feces. Anaerobic conditions were maintained in the growth media (thioglycollate, BioQuest) by bubbling nitrogen through the solution. Samples of the reaction mixture were taken at 4, 24, and 48 hr and distilled in a Packard oxidizer (Packard Instruments, Downers Grove, Ill.) for determination of tritium present as ³H₂O (15).

In order to determine the biologic stability of the label in man during enterohepatic cycling and intestinal transit, preliminary experiments were carried out in healthy subjects, after obtaining informed consent. [11,12-³H₂]Chenodeoxycholic acid (46 μ Ci) and [24-¹⁴C]chenodeoxycholic acid (10 μ Ci) were injected intravenously, and the ³H/¹⁴C ratio determined in bile as described previously (3). Feces were collected for 3 days, processed as previously described (15), and samples were oxidized using a Harvey biological oxidizer (R. J. Harvey Instrument Company, Hillsdale, NJ). Efficiency was calculated by combusting ³H- and ¹⁴C-labeled bile acid standards which had been mixed with powdered dried rabbit feces.

RESULTS AND DISCUSSION

Preparation of deuterium-labeled bile acids

For complete reduction of the olefin, acetic acid had to be used together with ethanol. Although reduction occurred nearly as rapidly in ethanol alone,

only two-thirds of the olefin was reduced and the addition of fresh catalyst still did not drive the reduction to completion. Further, traces of hydroxide ion normally present in commercial preparations of Adam's catalyst caused considerable hydrolysis of the ester groups during the course of the reaction. Homogeneous catalytic hydrogenation of olefins with Wilkinson's catalyst (tris(triphenylphosphine)chlororhodium) often gives saturated products with high isotopic purity (16), but with the bile acid olefin no reduction occurred, probably because of steric hindrance.

For a product of high isotopic purity, it was necessary to use deuterated solvents and to reduce the catalyst in situ. When reduction was carried out in acetone using prerduced Adam's catalyst (platinum dioxide, prepared by fusion of hexachloroplatinic acid and sodium acetate), the saturated product contained only 33% ²H₂ species. We attribute the low deuterium incorporation to traces of water adsorbed on the catalyst. McKenzie, Mattox, and Kendall (17) also reported that prerduced catalyst was ineffective in reducing the 3 α ,12 α -diacetoxy-5 β - $\Delta^{9,11}$ -cholenate.

This combination of deuterated ethanol-deuterated acetic acid with in situ catalyst reduction gave excellent reduction (100%) and resulted in a highly labeled product containing 75–85% dideutero species (7,18). Data presented in Table 3 summarize the isotope composition for a number of preparations of [11,12-²H₂]lithocholic acid and [11,12-²H₂]chenodeoxycholic acid prepared using the ethanol-acetic acid solvent.⁸ The lower yield of ²H species in lithocholic acid can be attributed to traces of protium-

⁸ An [11,12-²H₂]chenodeoxycholic acid product with still higher isotopic purity (96 to 98%) has been prepared using deuteration conditions developed at Merck, Sharp & Dohme, Canada Ltd., Montreal, Canada (I. Lesk and H. Koch, personal communication); this preparation is now available from the manufacturer.

TABLE 3. Isotope composition of representative samples of [11,12-²H]lithocholic acid and [11,12-²H]chenodeoxycholic acid prepared by selected method *a*

Compound	% ² H ₀ ^b	% ² H ₁ ^b	% ² H ₂ ^b
[11,12- ² H]Lithocholic acid			
Sample 1	7	27	66
Sample 2	15	18	67
[11,12- ² H]Chenodeoxycholic acid			
Sample 1	4	13	83
Sample 2	9	16	75

^a Unsaturated bile acids were reduced in (CH₃)₂ CHOD isopropanol with ²H₄-acetic acid (5:1, v/v) at room temperature until the theoretical amount of deuterium was absorbed.

^b The isotope composition data are corrected for the natural abundance of ¹³C contributed by ions at adjacent masses.

TABLE 4. Radiochemical purity of [11,12-³H]lithocholic and [11,12-³H]chenodeoxycholic acids estimated by cocrystallization with 24-¹⁴C-labeled bile acids

Crystallization	³ H/ ¹⁴ C ratio ^a	
	Chenodeoxycholic Acid	Lithocholic Acid
Before	1.00	1.00
1	0.97	1.03
2	0.99	1.02
3	0.99	1.00
4		1.01

^a Ratio before initial crystallization has been normalized to 1.00.

containing solvent of crystallization occluded in the crystal lattice of the unsaturated starting material. The low yield (36%) of [11,12-²H]chenodeoxycholic acid after deuteration of the di-acetate can be explained by incomplete hydrolysis at the 7 position (c.f. 14), by some loss during silica gel chromatography, and by a considerable loss during the crystallization procedure. When the reaction is carried out with greater mass, the yield should be much greater (>80%).

Preparation and radiochemical purity of tritium-labeled bile acids

The least exchange between solvent and olefin was obtained with platinum oxide in dioxane; these conditions afforded 62% reduction with 60% ²H isotopic purity. Thus, calculated specific activity of the tritiated product may be misleading in that the mass may include some of the olefin. However, the effect is small.

When [11,12-³H₂]chenodeoxycholic acid or [11,12-³H₂]lithocholic acid was cocrystallized with a ¹⁴C-labeled preparation of the same acid, radioactivity ratios remained constant (Table 4), thus confirming radiochemical purity of the tritiated chenodeoxycholic and lithocholic acids prepared by this method. Although this procedure indicates that label is present on the indicated bile acid, it provides no information on the amount of olefin present or the position of the tritium label.

Biologic stability

After incubation with a fecal homogenate, no radioactivity was detected in the distillate, suggesting stability of the label to bacterial enzymes, at least under these experimental conditions. After simultaneous administration of [11,12-³H]chenodeoxycholic acid and [24-¹⁴C]chenodeoxycholic acid to two healthy subjects, the ³H/¹⁴C ratio of bile was found to remain similar to that administered for 4 days. When the ³H/¹⁴C ratio of fecal radioactivity was determined, it was lower than that administered (Table 5),

suggesting some loss of the label during distal intestinal passage, presumably mediated by bacterial enzymes.

Value of labeling technique

The bile acid labeling procedure described here is fairly simple and rapid once the unsaturated precursor has been prepared; it is applicable to most types of bile acids. By dehydration of the 7 or 12 hydroxyl group and reduction with deuterium or tritium, it appears possible to produce labeled deoxycholic and ursodeoxycholic acids in addition to chenodeoxycholic and lithocholic acids. Since the method requires the sacrifice of a hydroxyl group, it cannot be used to produce a labeled cholic acid.

Although our data suggest that the 11,12-³H label in chenodeoxycholic acid is stable in healthy man during enterohepatic circulation, we believe that additional validation of this label is desirable before its extensive use in studies of bile acid metabolism in man. We have recently compared the behavior of the 11,12-²H label with that of the 24-¹⁴C label in chenodeoxycholic acid and found good agreement for estimates of bile acid pool size and synthesis rate determined by each nuclide (19). Still, the loss of ³H in the fecal sample suggests that [11,12-³H]chenodeoxycholic acid resembles [2,4-³H]chenodeoxycholic acid in being stable while in the exchangeable bile acid pool, but labile when in the bacteria-rich

TABLE 5. Stability of label in [11,12-³H]chenodeoxycholic acid^a

Subject	Bile ^b		Feces ^c	
	Day	³ H/ ¹⁴ C Ratio	Day	³ H/ ¹⁴ C Ratio
1	1	0.97	1	0.88
	2	0.96	2	0.88
	3	0.99	3	0.86
	4	0.99		
	5	0.95		
2	3	0.94		
	4	0.95		
	5	0.97		
3			1	0.81
			4	0.84
			5	0.83
4			3	0.81
			4	0.81
			5	0.80

^a [11,12-³H]Chenodeoxycholic acid (46 μCi) and [24-¹⁴C]chenodeoxycholic acid (10 μCi) were given intravenously and bile was collected from an indwelling tube. The ratio is expressed relative to that administered which has been normalized to 1.0.

^b Radioactivity was determined in bile without isolation of the chenodeoxycholic acid since it has been shown previously that virtually all of the radioactivity will be present as conjugates of chenodeoxycholic acid (22).

^c Feces were collected and kept refrigerated until homogenization. A 1.0-g aliquot was combusted using a commercial sample oxidizer as described in the text.

milieu of the distal intestine. Of course this label cannot be used in species that hydroxylate bile acids in position 12, but this has been observed only in the chicken (20). The [11,12-²H] bile acids would appear to be the best preparation of deuterium-labeled bile acids presently available, and should be useful for tracer studies of bile acid metabolism in pediatric and obstetric patients because radiation hazard is obviated (8,19).

In recent unpublished experiments,⁹ we have scrutinized the biologic stability of the [11,12-³H]label in lithocholic acid and found that the label may be lost in some individuals during enterohepatic cycling. If these observations are correct, [11,12-³H]lithocholic acid cannot be used for a valid estimate of bile acid kinetics in man by the isotope dilution technique. However, lithocholic acid has a unique enterohepatic circulation in that it is probably largely absorbed from the colon after deconjugation and desulfation (21), whereas most chenodeoxycholic acid is absorbed from the small intestine without deconjugation (22). Thus lability of the label in [11,12-³H]lithocholic acid may not imply lability of the label of [11,12-³H]chenodeoxycholic acid.

The biological stability of ³H bile acids prepared by the Wilzbach method also remains uncertain, since one preparation exhibited extensive loss of label (4), while another preparation, after extensive purification by partition chromatography, showed little and uniform loss when used in a small group of subjects (7). Thus, as emphasized by Panveliwalla, Pertsemlidis, and Ahrens (4), extensive validation of any preparation of ³H bile acid seems mandatory before its widespread application to the characterizing of bile acid metabolism can be accepted. ■■

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REFERENCES

- Hofmann, A. F., P. A. Szczepanik, and P. D. Klein. 1968. Rapid preparation of tritium-labeled bile acids by enolic exchange on basic alumina containing tritiated water. *J. Lipid Res.* **9**: 707-713.
- Hepner, G. W., J. A. Sturman, A. F. Hofmann, and P. J. Thomas. 1973. Metabolism of steroid and amino acid moieties of conjugated bile acids in man. III. Cholytaurine (taurocholic acid). *J. Clin. Invest.* **52**: 433-440.
- LaRusso, N. F., N. E. Hoffman, and A. F. Hofmann. 1974. Validity of using 2,4-³H-labeled bile acids to study bile acid kinetics in man. *J. Lab. Clin. Med.* **84**: 759-765.
- Panveliwalla, D. K., D. Pertsemlidis, and E. H. Ahrens, Jr. 1974. Tritiated bile acids: problems and recommendations. *J. Lipid Res.* **15**: 530-532.
- Ståhl, E., and B. Arnesjö. 1972. Taurocholate metabolism in man. *Scand. J. Gastroenterol.* **7**: 559-566.
- Wilzbach, K. E. 1957. Tritium-labeling by exposure of organic compounds to tritium gas (letter to the editor). *J. Amer. Chem. Soc.* **79**: 1013.
- Einarsson, K., K. Hellström, and M. Kallner. 1974. Randomly tritium-labelled chenodeoxycholic acid as tracer in the determination of bile acid turnover in man. *Clin. Chim. Acta.* **56**: 235-239.
- Watkins, J. B., D. Ingall, P. Szczepanik, P. D. Klein, and R. Lester. 1973. Bile-salt metabolism in the newborn: measurement of pool size and synthesis by stable isotope technic. *N. Engl. J. Med.* **288**: 431-434.
- Hachey, D. L., P. A. Szczepanik, O. W. Berngruber, and P. D. Klein. 1973. Syntheses with stable isotopes: synthesis of deuterium and ¹³C-labeled bile acids. *J. Labelled Compd.* **9**: 703-719.
- Vroman, H. E., and C. F. Cohen. 1967. Separation of sterol acetates by column and thin-layer argentation chromatography. *J. Lipid Res.* **8**: 150-152.
- Nakada, F., and K. Yamasaki. 1963. Dehydration of bile acids and their derivatives. XI. A novel route to 3 α -hydroxychol-11-enic acid from deoxycholic acid. *Steroids.* **1**: 131-135.
- Nakada, F. 1963. Dehydration of bile acids and their derivatives. XII. Chola-3,7,11-trienic acid and its partially hydrogenated compounds—a note on α -cholatrienic acid II. *Steroids.* **2**: 45-59.
- Fieser, L. F., and S. Rajagopalan. 1950. Oxidation of steroids. III. Selective oxidations and acylations in the bile acid series. *J. Amer. Chem. Soc.* **72**: 5530-5536.
- Hauser, E., E. Baumgartner, and K. Meyer. 1960. Zur Kenntnis der Chenodesoxycholsäure (3 α ,7 α -dihydroxy-5 β -cholansäure). *Helv. Chim. Acta.* **43**: 1595-1600.
- Fromm, H., P. J. Thomas, and A. F. Hofmann. 1973. Sensitivity and specificity in tests of distal ileal function: prospective comparison of bile acid and vitamin B₁₂ absorption in ileal resection patients. *Gastroenterology.* **64**: 1077-1090.
- Hussey, A. S., and Y. Takeuchi. 1969. Hydrogenations of cycloalkenes using tris(triphenylphosphine)chlororhodium(I). *J. Amer. Chem. Soc.* **91**: 672-675.
- McKenzie, B. F., V. R. Mattox, and E. C. Kendall. 1948. Steroids derived from bile acids. VIII. Catalytic hydrogenation of methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholenate and related compounds. *J. Biol. Chem.* **175**: 249-263.
- Klein, P. D., D. L. Hachey, P. A. Szczepanik, and A. F. Hofmann. 1975. Synthesis of ²H- and ¹³C-labeled bile acids and their analysis by electron impact and chemical

⁹ Allan, R. and A. F. Hofmann, unpublished observations.

ionization mass spectrometry. *In* Proceedings of the Third Bile Acid Meeting, S. Matern, P. Back, and J. Hackenschmidt, editors. Schattauer Verlag, Frankfurt. 39-44.

19. Balistreri, W. F., A. E. Cowen, A. F. Hofmann, P. A. Szczepanik, and P. D. Klein. 1975. Validation of use of 11,12-²H-labeled chenodeoxycholate in isotope dilution measurements of bile acid kinetics in man. *Pediatr. Res.* **9**: 757-760.
20. Palmer, R. H. 1972. Bile acids, liver injury and liver disease. *Arch. Intern. Med.* **130**: 606-617.
21. Cowen, A. E., M. G. Korman, A. F. Hofmann, O. W. Cass, and S. B. Coffin. 1975. Metabolism of lithocholate in healthy man. II. Enterohepatic circulation. *Gastroenterology.* **69**: 67-76.
22. Hepner, G. W., A. F. Hofmann, and P. J. Thomas. 1972. Metabolism of steroid and amino acid moieties of conjugated bile acids in man. II. Glycine-conjugated dihydroxy bile acids. *J. Clin. Invest.* **51**: 1898-1905.