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# Bile Acids. XXVI. The Metabolism of 12α-Hydroxycholanoic Acid-24-$^{14}$C in the Rat*

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ABSTRACT: 12α-Hydroxycholanoic acid-24-$^{14}$C was prepared by a nitrile synthesis from the norbromide and administered intraperitoneally to each of three rats with bile fistulas. Within 24 hr most of the administered $^{14}$C was recovered in bile. After alkaline hydrolysis of the conjugated bile acids, the free bile acids were separated by partition chromatography. Of the chromatographed $^{14}$C, 12% was identified as unchanged 12α-hydroxycholanoic acid, 26% as 7α,12α-dihydroxycholanoic acid, 18% as deoxycholic acid, 15% as cholic acid, and a small amount of 6β,12α-dihydroxycholanoic acid, a new bile acid. Methods of preparation and data for characterization of the new acid are provided. These results extend earlier observations on the ability of the rat to oxygenate the nucleus of cholanoic acid at position 7 in the absence of a 3α-hydroxyl group and suggest a limited ability to oxygenate 12α-hydroxycholanoic acid in the 6β position.

The ability of the rat to hydroxylate bile acids at positions 6β and 7α has been demonstrated in several previous studies. For example, lithocholic acid is metabolized to chenodeoxycholic acid, 3α,6β-dihydroxycholanoic acid, and α- and β-muricholic acids (Thomas *et al.*, 1964); and deoxycholic acid is metabolized mainly to cholic acid (Bergström *et al.*, 1953) with 3α,6β,12α-trihydroxycholanoic acid as a minor product (Ratliff *et al.*, 1959).

Hydroxylation at positions 6β and 7α does not seem to be limited to the natural occurring bile acids which contain hydroxyl groups at position 3α. In a previous study at this laboratory cholanoic acid was found to form 7α-hydroxycholanoic, chenodeoxycholic, and α- and β-muricholic acids. Since lithocholic acid was not detected among the metabolites, the identification of 7α-hydroxycholanoic acid suggested that the metabolic process might be initiated by 7α hydroxylation rather than 3α hydroxylation (Ray *et al.*, 1961). In an attempt to determine what effects, if any, hydroxylation at the 12α position of the steroid nucleus may have on the course of bile acid metabolism, we have studied the metabolism of 12α-hydroxycholanoic acid in the rat. This study necessitated the preparation of a series of new bile acids. Methods of preparation and data for characterization of these new acids are also included with results of metabolic studies in this report.

## Experimental Procedure[1]

*Chromatography.* Bile acids were chromatographed on an acetic acid partition column as previously described (Matschiner *et al.*, 1957). The fractions have been designated according to the percentage of benzene in hexane. For example, fraction 20-1 represents the first fraction of the eluent containing 20% benzene in hexane. The procedure of reversed-phase partition column chromatography of Bergström and Sjövall

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[1] Melting point determinations were taken on a Fisher-Johns apparatus and are reported as read. Infrared spectra were determined in Nujol with a Perkin-Elmer spectrophotometer, Model 21, equipped with rock salt optics and ordinate scale expansion. Ultraviolet spectra were determined with a Hitachi Perkin-Elmer spectrophotometer, Model 139. Optical rotations were determined in methanol at a concentration of 1% unless stated otherwise with a Rudolph photoelectric spectropolarimeter, Model 200-S. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Radioactivity was measured with a Packard Tri-Carb liquid-scintillation spectrometer, Model 3314, as previously reported (Ray *et al.*, 1961).

(1959), as modified by Ray *et al.* (1961), was used for the separation of the methyl esters of monohydroxy- and monoketocholanoic acids. Chromatography on silica gel was employed in the purification of large quantities of the methyl esters of bile acids. Silica gel (Grace Davidson Chemical Co.; mesh 100–200) was washed with methanol and air dried under suction for 24 hr. The ratio of silica gel to sample was 30:1. Numerous solvent systems of elution were used in accord with the polarity of the compound. Adsorption chromatography on alumina was used in the purification of the norbromide. The alumina (Fisher Scientific; mesh 80–200) was treated with acetic acid, washed with methanol, and dried at 100° for 1 hr. Plates for thin-layer chromatography (5 × 20 or 20 × 20 cm) were prepared with silica gel G or H (Brinkmann Instruments Inc.) by the method of Eneroth (1963).

Gas chromatography of methyl esters of bile acids and their derivatives was carried out on a Barber-Colman gas chromatograph, Model 10, with an argon–$^{90}$Sr detector and silanized glass columns (2 m × 6 mm) packed with 1% SE-30, 1% QF-1, or 1% SE-52 on siliconized Celite[2] (100–200 mesh); flash heater, 290°; column oven, 223°; and argon flow at exit, 30–150 cc/min.

*Preparation of 12α-Hydroxycholanoic Acid-24-$^{14}$C.* Methyl 3-keto-12α-hydroxycholanoate (mp 140–142°) was obtained from methyl deoxycholate according to the procedure of Jones *et al.* (1949). After saponification of this ester, the keto acid (mp 118–119°) was treated with alkaline hydrazine in a Huang-Minlon modification of the Wolff–Kishner method according to Mosbach *et al.* (1954). 12α-Hydroxycholanoic acid formed white needles from acetic acid: mp 90–93°, $[\alpha]_D^{25}$ +41.9 ± 0.5°; lit. (Sorkin and Reichstein, 1943) mp 90–95°. Elemental analysis indicated solvation of the crystalline material[3] as reported by Barnett and Reichstein (1938) and Wieland and Schlichting (1925).

12α-Hydroxycholanoic acid (2.6 g) was acetylated in the usual manner to provide 2.2 g of crude product. Purification by preparative thin-layer chromatography and crystallization from a mixture of acetone and water provided needles: mp 208–209°, $[\alpha]_D^{25}$ +61.5 ± 0.1° ($CHCl_3$, *c* 0.5%); lit. (McIntosh *et al.*, 1949) mp 214–215°, $[\alpha]_D^{25}$ + 71°.

The norbromide was prepared by bromine degradation of the silver salt of 12α-acetoxycholanoic acid (2.63 g) according to the procedure of Brink *et al.* (1946). The norbromide was chromatographed on alumina (50 g) and the oil which eluted with hexane crystallized from hot methanol: mp 160–161°, $[\alpha]_D^{25}$ +79.3 ± 1° ($CHCl_3$; *c* 1.0). *Anal.* Calcd for $C_{25}H_{41}O_3Br$: C, 66.21; H, 9.11; Br, 17.62. Found: C, 66.10; H, 9.15; Br, 17.37.

12α-Hydroxycholanoic acid-24-$^{14}$C was prepared from 12α-acetoxy-24-norbromocholane by a nitrile synthesis (Mahowald *et al.*, 1957) with 0.5 mCi of [$^{14}$C]KCN (Nuclear-Chicago Corp.) After purification by chromatography on a mixture of Celite and silicic acid (1:1) and crystallization to constant specific activity, the material had a melting point of 90–93° and a specific activity of 1.24 × $10^7$ dpm per mg. The radiopurity of the acid was confirmed by isotopic dilution of the methyl ester; after seven successive crystallizations from aqueous methanol the specific activity varied less than 2% from the calculated value. Authentic methyl 12α-hydroxycholanoate showed by gas chromatography on 1% SE-30 a single peak with retention time of 3.39 min. Similar amounts of the radioactive ester had identical retention time, and overloading the column with larger quantities gave evidence that only one component was present.

*Preparation of Reference Compounds.* 1. 7α,12α-Dihydroxycholanoic acid. This material was prepared from methyl cholate *via* Oppenauer oxidation to 3-keto-7α,12α-dihydroxycholanoic acid and Huang-Minlon reduction of this product. Purification by partition chromatography and crystallization from a mixture of ethyl acetate and hexane provided needles at mp 212–213° and $[\alpha]_D^{25}$ +28 ± 1° in agreement with the reports of Jones *et al.* (1949), Pietra and Traverso (1951), and Grand and Reichstein (1945).

Treatment of 7α,12α-dihydroxycholanoic acid with a solution of 1% methanolic HCl afforded methyl 7α,12α-dihydroxycholanoate (I), mp 156° (lit. mp 154°) (Jones *et al.*, 1949).

2. 6β,12α-Dihydroxycholanoic acid (Chart I). A. Methyl 7-Keto-12α-hydroxycholanoate (II). The oxidation of methyl 7α,12α-dihydroxycholanoate (I) was carried out in a manner analogous to that described by Haslewood (1943). From 12.67 g of methyl 7α,12α-dihydroxycholanoate 10.8 g of crude methyl 7-keto-12α-hydroxycholanoate were obtained, mp 130°. The ester was purified by chromatography on a column of silica gel and was eluted with a mixture of acetone–benzene–hexane (8:46:46); after crystallization from aqueous methanol, 8.5 g was obtained (mp 140° and $[\alpha]_D^{25}$ −17.1°); on thin-layer chromatography with 10% acetone in benzene only one spot appeared after development with phosphomolybdic acid: infrared spectrum 3521 (hydroxyl), 3425 (hydroxyl), 1742 (methyl ester), 1707 (7-keto), 1252, 1171 (methyl ester), 1095, 1074, 1040, 1017, 941, 842, and 751 $cm^{-1}$. *Anal.* Calcd for $C_{25}H_{40}O_4$: C, 74.22; H, 9.96. Found: C, 74.09; H, 9.81.

B. Methyl 7-Keto-12α-acetoxycholanoate (III). The procedure of Turner (1953) was used for acetylation. Methyl 7-keto-12α-hydroxycholanoate (II) (8 g) stood overnight at room temperature in a solution of 160 ml of glacial acetic acid, 32 ml of acetyl chloride, and 8 g of *p*-toluenesulfonic acid. Crystallization of the product from aqueous methanol provided 7.4 g of methyl 7-

[2] SE-30 is a commercially available methylsilicone polymer; QF-1 is a commercially available fluorosilicone, and SE-52 is a commercially available methylphenylsilicone polymer.

[3] In order to characterize this solvated product the following derivatives were prepared: methyl 12α-hydroxycholanoate (mp 119–120°, $[\alpha]_D^{25}$ + 44.9 ± 1°; lit. (Barnett and Reichstein, 1938) mp 118–119°, $[\alpha]_D^{25}$ +39.5°), 12-ketocholanoic acid (mp 187–189°; lit. (Hoehn and Linsk, 1945) mp 187–189°), and methyl 12-ketocholanoate (mp 109, $[\alpha]_D^{25}$ +96.5 ± 1°; lit. (Alther and Reichstein, 1942) mp 108–109°, $[\alpha]_D^{25}$ +88°). Elemental analyses of these compounds were in agreement with calculated values.

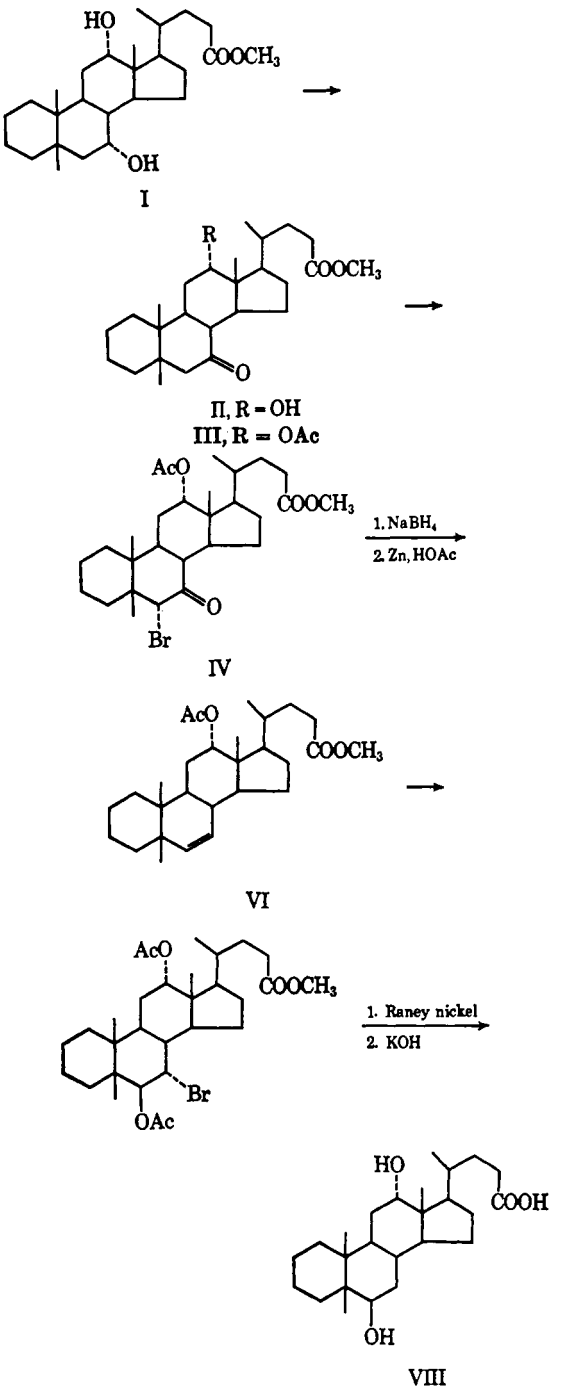
keto-12α-acetoxycholanoate, mp 113–114°. A sample was purified by preparative thin-layer chromatography on silica gel H with 8% acetone in benzene. The region of the silica gel containing the ester was scraped from the plate, washed with acetone and ethyl acetate, and the washings were taken to dryness. The residue was crystallized from a mixture of methanol and water: mp 114°; $[\alpha]_D^{25}$ +27.2 ± 0.5°; infrared 1730 (methyl ester), 1715 (7-keto), 1239 (acetate), 1168, 1115 (weak), 1093 (weak), 1070, 1030, 1015 (weak), 955, 901, and 839 $cm^{-1}$; ultraviolet spectra (max) (95% $C_2H_5OH$) 289 mμ (log ε 1.39). *Anal.* Calcd for $C_{27}H_{42}O_5$: C, 72.61; H, 9.48. Found: C, 72.32; H, 9.33.

C. Methyl 6α-Bromo-7-keto-12α-acetoxycholanoate (IV). The bromination of methyl 7-keto-12α-acetoxycholanoate (III) was conducted by a procedure analogous to that described by Takeda *et al.* (1954). A solution of 7.72 g of methyl 7-keto-12α-acetoxycholanoate in 85 ml of glacial acetic acid was stirred at 25° during dropwise addition of 2.75 g of bromine in 1.5 ml of glacial acetic acid and 2.47 ml of 30% HBr in glacial acetic acid. The mixture was allowed to stand at room temperature for 2 hr, diluted with 500 ml of water, and extracted with diethyl ether. The residue from the ether extract was crystallized from aqueous methanol to provide 7.91 g of methyl 6α-bromo-7-keto-12α-acetoxycholanoate: mp 165°; $[\alpha]_D^{25}$ +4.4 ± 0.5°; infrared 1739 (sh), 1730 (methyl ester), 1252 (acetate), 1109, 1082 (weak), 1059, 1026, 1010 (weak), 963, 894, 833, 760, and 749 $cm^{-1}$; ultraviolet (max) (95% $C_2H_5$-OH) 281 mμ (log ε 1.46). *Anal.* Calcd for $C_{27}H_{41}BrO_5$: C, 61.71; H, 7.86; Br, 15.21. Found: C, 61.85; H, 7.92; Br, 15.1.

D. Methyl 6α-Bromo-7α-hydroxy-12α-acetoxycholanoate (V). Methyl 6α-bromo-7-keto-12α-acetoxycholanoate (5.67 g) was dissolved in 225 ml of methanol, the mixture was cooled to 5° in an ice bath, and 16 g of sodium borohydride was added slowly. The reaction mixture was kept at ice-bath temperature until the evolution of gas ceased, left at room temperature for 1 hr, and then acidified with dilute acetic acid. The solution was evaporated to a small volume at room temperature, diluted with water, and extracted with ether. The residue was crystallized from a mixture of methanol and water to provide 4.74 g of methyl 6α-bromo-7α-hydroxy-12α-acetoxycholanoate:[4] mp 141–143°; $[\alpha]_D^{25}$ +45.8 ± 1°; infrared 3534 (hydroxyl), 1745 (methyl ester, acetate), 1248 (acetate), 1182 (methyl ester), 1129, 1109, 1085, 1070, 1026 (hydroxyl), 979, 960, 926, 907, 884, 855, 816, 796, 773, 742, 711, and 691 $cm^{-1}$. *Anal.* Calcd for $C_{27}H_{43}BrO_5$: C, 61.47; H, 8.22; Br, 15.15. Found: C, 61.21; H, 8.18; Br, 15.03.

E. Methyl 12α-Acetoxy-Δ⁶-cholenoate (VI). This unsaturated compound was prepared by refluxing 5.64 g of the bromohydrin (V) in 100 ml of glacial acetic acid during the slow addition of 12 g of zinc dust over a period of 30 min (Hsia *et al.*, 1957). After the mixture was refluxed for an additional 2 hr, the zinc dust was removed by filtration, the filtrate was diluted

CHART I




[4] Chromatography of IV on silica gel provided another bromo ketone: mp 155° from aqueous methanol, $[\alpha]_D^{25}$ +4.3°; ultraviolet spectrum max 277 mμ; infrared spectrum comparable with V. Reduction of this derivative with $NaBH_4$ provided a bromohydrin: mp 125° (aqueous methanol), $[\alpha]_D^{25}$ +45.7°; infrared spectrum comparable to V. Treatment of this lower melting bromohydrin with zinc and acetic acid afforded a product identical in all respects with VI. Elemental analyses were consistent with the assignment of structure as monobromo ketone and monobromohydrin, respectively. These derivatives may represent different crystalline forms of IV and V.

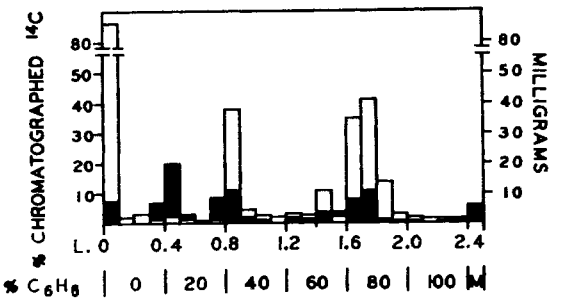



FIGURE 1: Chromatographic analysis of the free bile acids from bile of rat A collected during the first 2 days. The heights of the open bars from the base line to the top indicate amount of eluted solids. The heights of the solid bars from the base line to the top indicate the percentage of chromatographed $^{14}C$ eluted. The composition of the eluent (per cent $C_6H_6$) is given as the percentage of benzene in hexane. The volume of eluent is given in liters (L). The fraction designated M represents the methanol wash.

with water and extracted with ether; the ether layer was evaporated to dryness. The residue (4.73 g) was chromatographed on a column of silica gel and eluted with 10% benzene in hexane; 3.75 g of methyl 12$\alpha$-acetoxy-$\Delta^6$-cholenoate was crystallized from aqueous methanol, mp 82°.

A sample was purified by preparative thin-layer chromatography on silica gel H with 1% acetone in benzene. The product was crystallized from aqueous methanol: mp 82°; $[\alpha]_D^{25}$ +36.2 ± 1°; infrared 1742 (methyl ester, acetate), 1318, 1244 (acetate), 1195, 1166 (methyl ester), 1089, 1067, 1025, 987, 966, 948, 910, 873, 848, 771, 754, 736, and 708 $cm^{-1}$. *Anal.* Calcd for $C_{27}H_{42}O_4$: C, 75.31; H, 9.83. Found: C, 75.09; H, 9.85.

A sample of methyl 12$\alpha$-acetoxy-$\Delta^6$-cholenoate was reduced catalytically in the presence of platinum and the residue was hydrolyzed with methanolic KOH. The crystals of 12$\alpha$-acetoxycholanoic acid (mp 208–210°) showed no depression in melting point on admixture with the authentic acid.

An epoxide (VIa) was formed by the addition of an excess of monoperphthalic acid to a solution of 500 mg of methyl 12$\alpha$-acetoxy-$\Delta^6$-cholenoate in ether (Hsia *et al.*, 1957). After standing at room temperature for 3 days the reaction mixture was extracted with dilute sodium carbonate solution and washed with water; the ether solution was evaporated to dryness and the residue was crystallized from aqueous methanol as 430 mg of colorless needles: mp 114°; $[\alpha]_D^{25}$ +48.8 ± 1°; infrared 1742 (methyl ester, acetate), 1312 (weak), 1242 (acetate), 1166 (methyl ester), 1122, 1099, 1071, 1020 (br), 965, 937, 876, 856, 831, 826, 801, 780, 737, and 703 $cm^{-1}$. Samples of this preparation exhibited single peaks in gas–liquid partition and thin-layer chromatography. *Anal.* Calcd for $C_{27}H_{42}O_5$: C, 72.61; H, 9.48. Found: C, 72.36; H, 9.40.

F. Methyl 6$\beta$,12$\alpha$-Diacetoxy-7$\alpha$-bromocholanoate (VII). To a suspension of finely pulverized silver acetate (2.6 g) in 60 ml of glacial acetic acid, 2.5 g of bromine was added slowly with stirring (Ratliff *et al.*, 1961). The faintly yellow supernatant solution was added with stirring to a solution of 4.5 g of methyl 12$\alpha$-acetoxy-$\Delta^6$-cholenoate (VI) in 70 ml of glacial acetic acid. The mixture was stirred with a magnetic stirrer for an additional 30 min, diluted with water, and extracted with ether. The ether extract was evaporated to dryness and the residue was chromatographed on a column of silica gel. Methyl 6$\beta$,12$\alpha$-diacetoxy-7$\alpha$-bromocholanoate (2.49 g) was eluted with 60% benzene in hexane, and the product was crystallized from a mixture of methanol and water, mp 104°. A sample was further purified by preparative thin-layer chromatography on silica gel H with 1% acetone in benzene. The region of the silica gel containing the ester was scraped from the plate and washed with acetone and ethyl acetate, and the washings were evaporated to dryness. The residue was crystallized from aqueous methanol: mp 104°; $[\alpha]_D^{25}$ +45.3 ± 0.5° ($CHCl_3$, *c* 1%); infrared spectrum 1739 (methyl ester, acetate), 1318, 1233 (acetate), 1192, 1172 (methyl ester), 1109, 1070, 1026, 971, 899, and 690 $cm^{-1}$. *Anal.* Calcd for $C_{29}H_{45}BrO_6$: C, 61.15; H, 7.96; Br 14.03. Found: C, 61.29; H, 8.01; Br, 14.06.

G. 6$\beta$,12$\alpha$-Dihydroxycholanoic Acid (VIII). Methyl 6$\beta$,12$\alpha$-diacetoxy-7$\alpha$-bromocholanoate (1 g) was debrominated by refluxing for 18 hr with 10 g of W-2 Raney nickel (Mozingo *et al.*, 1943) in a mixture of 100 ml of ethanol, 40 ml of water, and 13 drops of glacial acetic acid. The catalyst was removed by filtration, the filtrate was concentrated, and the residue was hydrolyzed with 5% methanolic KOH in an autoclave for 2.5 hr. The product was chromatographed on a partition column; 366 mg of 6$\beta$,12$\alpha$-dihydroxycholanoic acid was obtained from fractions 20-2 and 20-3 and crystallized from aqueous methanol, mp 119°. A sample of the acid was purified by preparative thin-layer chromatography with a mixture of 50 ml of isooctane, 50 ml of ethyl acetate, and 0.7 ml of glacial acetic acid. The acid obtained from this procedure was crystallized from aqueous methanol: mp 119°; $[\alpha]_D^{25}$ +44.4 ± 0.5°; infrared spectrum 3401 (hydroxyl), 1712 (carboxyl), 1029, 1014 (weak), 956, and 869 $cm^{-1}$. *Anal.* Calcd for $C_{24}H_{40}O_4$: C, 73.43; H, 10.27. Found: C, 73.16; H, 10.33.

The methyl ester of this acid was prepared with diazomethane in the usual manner, chromatographed on a partition column, and eluted in fraction 0-4. The ester has not been crystallized.

H. 6$\beta$-Hydroxycholanoic Acid (X). To ascertain the stereochemistry of the hydroxyl at position 6 in the above product (VIII), the 12$\alpha$-hydroxyl group was removed by the following procedure.

The residue from treatment of VIII with diazomethane was acetylated in the usual manner with a mixture of acetic anhydride and pyridine and the product was chromatographed on silica gel. After elution with 4% acetone in benzene, the product was purified by preparative layer chromatography with 4% acetone in benzene. By repetition of this procedure 171 mg of monoacetate was obtained; this material was oxidized in 5 ml of acetic acid with a mixture of 60 mg of $CrO_3$ in 4 ml of $H_2O$. The product was extracted with ether and the residue (169 mg) which remained after evaporation of the ether was saponified with 5% aqueous

KOH for 1 hr in an autoclave at 122°. After acidification and extraction with ether, 154 mg of crystalline material was obtained from the ether. After crystallization from a mixture of acetone and hexane, a portion of the crystals (34 mg) was purified by preparative thin-layer chromatography with a mixture of ethyl acetate, isooctane, and acetic acid (55:45:1) to provide 25.3 mg of needles of 6β-hydroxy-12-ketocholanoic acid (IX): mp 196°; $[\alpha]_D^{25}$ +88.1°; infrared spectrum 3356, 1721, 1684, 1174, 1101, 1041, 910, 805, 757, and 678 $cm^{-1}$.

A Wolff–Kishner reaction (Huang-Minlon, 1949) was carried out on 68 mg of IX, and the product (50 mg) was purified by partition chromatography. From fraction 0-1 through 0-3, 48.1 mg of residue (X) was obtained which was crystallized from a mixture of acetone and hexane (mp 183°, $[\alpha]_D^{25}$ +31 ± 1°); this product was identical with 6β-hydroxycholanoic acid obtained previously by reduction of 6-ketocholanoic acid with $NaBH_4$ (Ray, 1962).

*Metabolic Studies.* Each of three adult male rats (310 g) of the St. Louis University colony with a cannulated bile duct was placed in a restraining cage with free access to Purina Lab Chow, water, and a solution of 1.5% sodium bicarbonate and 0.6% NaCl. Every third day the diets were amply supplemented orally with water-solubilized fat-soluble vitamins A, D, E, and K. After a postoperative period of 24 hr, each rat was injected intraperitoneally with approximately 1 mg of 12α-hydroxycholanoic acid-24-$^{14}C$ ($1.24 \times 10^7$ dpm) as the potassium salt in a 20% alcoholic solution. Urine and bile samples were collected daily and assayed for radioactivity. Most of the $^{14}C$ was recovered in the bile during the first 24 hr; less than 0.5% was recovered after the first day, and less than 0.7% was found in urine.

## Results

*Fractionation of Radioactive Metabolites in the Bile.* Bile from the first 2 days of rat A contained about 94% of the administered radioactivity. After saponification of the conjugated bile acids according to the procedure of Mahowald *et al.* (1957) approximately 87% of the $^{14}C$ was present in the acidic fraction and 13% remained water soluble. The free bile acids were separated on a partition column. The chromatogram (Figure 1, rat A) shows that 7.9% of the chromatographed $^{14}C$ appeared in the monohydroxy acid fraction (fraction 0-1), 29.1 and 21.5% appeared in each of two peaks in the dihydroxy acid fractions (fractions 0-4, 20-1, and 20-2, and fractions 20-4, 40-1, and 40-2, respectively), 21.6% appeared in fractions associated with cholic acid (fractions 60-4, 80-1, and 80-2), and 7.8% appeared in fractions less hydrophilic than cholic acid (fractions 60-1, 60-2, and 60-3).

Comparable results were obtained from a second animal (rat B); 12.1% of the chromatographed $^{14}C$ was retained in the monohydroxy acid fraction, 53.1% in the dihydroxy acid fractions, and 25.2% in the fractions associated with cholic acid.

*Investigation of Fraction 0-1.* An aliquot of fraction 0-1 ($1.29 \times 10^5$ dpm) from rat B was diluted with authentic methyl 12α-hydroxycholanoate and the mixture was methylated with diazomethane; the product was chromatographed on a reversed-phase partition column. The methyl ester in fractions 9 through 12 was combined and crystallized four times from a mixture of methanol and water with a change of ±1% in specific activity from the calculated value.

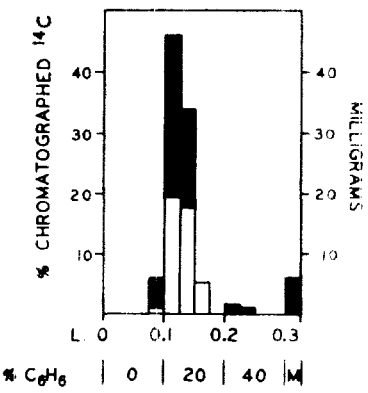



FIGURE 2: Chromatogram of a mixture of 7α,12α-dihydroxycholanoic acid and portion of the $^{14}C$ from fractions 0-4 through 20-2 (Figure 1). The heights of the open bars from the base line to the top indicate amount of eluted solids. The heights of the solid bars from the base line to the top indicate the percentage of chromatographed $^{14}C$ eluted. The composition of the eluent (per cent $C_6H_6$) is given as the percentage of benzene in hexane. The volume of eluent is given in liters (L). The last fraction (M) represents the methanol wash.

After hydrolysis of the methyl ester, the free acid was chromatographed on a reversed-phase partition column, and the eluted acid was crystallized twice from glacial acetic acid. From the specific activities of the ester and the free acid, it was calculated that 97.6% of the $^{14}C$ in fraction 0-1 was identified as 12α-hydroxycholanoic acid; this is equivalent to 11.8% of the chromatographed $^{14}C$ (rat B).

*Identification of 7α,12α-Dihydroxycholanoic Acid.* Fractions 0-4 through 20-2 (Figure 1) were combined and represented 29.1% of the chromatographed radioactivity. An aliquot ($2.90 \times 10^5$ dpm) was diluted with 48.9 mg of authentic 7α,12α-dihydroxycholanoic acid and chromatographed on a partition column. The radioactivity was eluted coincidentally with the mass (Figure 2) and the fractions containing 7α,12α-dihydroxycholanoic acid were combined and crystallized to a constant specific activity (Table I). The remaining crystals were methylated with diazomethane, chromatographed on a partition column, and the methyl ester from fractions 0-1 and 0-2 was combined and crystallized. The specific activity remained constant after several crystallizations from various solvent systems (Table I). From the specific activities, it was calculated that 79% of the radioactivity was present in 7α,12α-dihydroxycholanoic acid; this is equivalent to 23% of the chromatographed $^{14}C$ (Figure 1).

In rat B, an aliquot from the dihydroxy zone (fractions 0-4 through 40-3) representing 53.1% of the chromatographed $^{14}C$ was diluted with authentic 7α,12α-dihydroxycholanoic acid and treated similarly. From

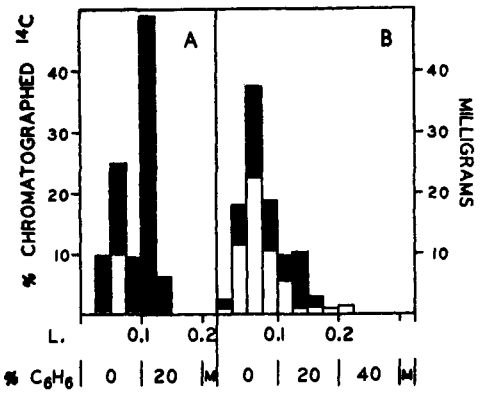



FIGURE 3: Chromatographic studies. (A) Of a mixture of methyl 6β,12α-dihydroxycholanoate and a portion of the $^{14}C$ from fractions 20-3 and 20-4 (Figure 1) after treatment with diazomethane. (B) Of a mixture of methyl deoxycholate and a portion of the $^{14}C$ from fractions 20-3 through 40-3 (Figure 1) after treatment with diazomethane.

In each chromatogram the heights of the open bars from the base line to the top indicate amount of eluted solids. The heights of the solid bars from the base line to the top indicate the percentage of chromatographed $^{14}C$ eluted. The composition of the eluent (per cent $C_6H_6$) is given as the percentage of benzene in hexane. The volume of eluent is given in liters (L). The last fraction (M) represents the methanol wash.

the specific activities of the acid and the methyl ester, 49.4% of the radioactivity in the dihydroxy zone was present in 7α,12α-dihydroxycholanoic acid, which is equivalent to 26.2% of the chromatographed $^{14}C$ (rat B).

*Identification of 6β,12α-Dihydroxycholanoic Acid.* From fractions 0-4 through 40-3 derived from rat B, an aliquot ($5.76 \times 10^5$ dpm) was diluted with 23.8 mg of 6β,12α-dihydroxycholanoic acid and chromatographed on a partition column. The mass was eluted in fractions 20-3 and 20-4 and represented 14% of the $^{14}C$ in the dihydroxy zone. These fractions were combined and crystallized, and specific activities were determined (Table II). The remaining crystals were methylated with diazomethane and chromatographed on a partition column (Figure 3A). The major portion of the mass was eluted in fraction 0-3, whereas more than half of the chromatographed $^{14}C$ was eluted in fractions more polar than methyl 6β,12α-dihydroxycholanoate. Since the ester could not be crystallized, it was hydrolyzed in methanolic KOH to the free acid and the acid was purified by partition chromatography. The residue from fraction 20-2 containing 6β,12α-dihydroxycholanoic acid was crystallized from aqueous methanol and specific activities were determined (Table II). From the final specific activity, it was calculated that at mos

TABLE II: Identification of 6β,12α-Dihydroxycholanoic Acid[a] in Bile of Rat B.

| Solvent | Crystzn | Amt (mg) | Sp Act. (dpm/mg × $10^{-3}$) |
|---|---|---|---|
| Free acid | | | |
| Methanol + $H_2O$ | 2 | 18.9 | 3.55 |
| Methanol + $H_2O$ | 2 | 15.2 | 2.18 |
| Free acid from ester | | | |
| Methanol + $H_2O$ | 2 | 6.8 | 0.39 |
| Methanol + $H_2O$ | 2 | 3.1 | 0.34 |

[a] 6β,12α-Dihydroxycholanoic acid (23.852 mg) was added to $5.76 \times 10^5$ dpm to give an original specific activity of $2.42 \times 10^4$ dpm per mg.

TABLE I: Identification of 7α,12α-Dihydroxycholanoic Acid[a] in Bile of Rat A.

| Solvent | Crystzn | Amt (mg) | Sp Act. (dpm/mg × $10^{-3}$) |
|---|---|---|---|
| Free acid | | | |
| Ethyl acetate | 2 | 42.1 | 4.94 |
| Acetone + $H_2O$ | 2 | 32.6 | 4.78 |
| Ethyl acetate + hexane | 2 | 28.4 | 4.75 |
| Methyl ester | | | |
| Methanol + $H_2O$ | 2 | 22.9 | 4.71 |
| Acetone + $H_2O$ | 2 | 16.1 | 4.70 |
| Acetone + hexane | 2 | 6.4 | 4.68 |

[a] 7α,12α-Dihydroxycholanoic acid (48.914 mg) was added to $2.90 \times 10^5$ dpm to give an original specific activity of $5.92 \times 10^3$ dpm per mg.

TABLE III: Identification of Deoxycholic Acid[a] in Bilet of Rat A.

| Solvent | Crystzn | Amt (mg) | Sp Act. (dpm/mg × $10^{-3}$) |
|---|---|---|---|
| Methyl ester | | | |
| Acetic acid + $H_2O$ | 3 | 34.2 | 4.56 |
| Acetic acid + $H_2O$ | 2 | 23.0 | 4.37 |
| Methyl ester diacetate | | | |
| Methanol + $H_2O$ | 2 | 17.6 | 4.42 |
| Methanol + $H_2O$ | 2 | 12.0 | 4.33 |
| Methanol + $H_2O$ | 2 | 8.3 | 4.35 |

[a] Methyl deoxycholate (46.823 mg) was added to $2.56 \times 10^5$ dpm to give an original specific activity of $5.66 \times 10^3$ dpm per mg. The specific activities of methyl deoxycholate and the methyl 3α,7α-diacetoxycholanoate are corrected on a molar basis to the free acid.

TABLE IV: Identification of Cholic Acid[a] in Bile of Rat A by Isolation.

| Solvent | Crystzn | Amt (mg) | Sp Act. (dpm/mg × $10^{-4}$) |
|---|---|---|---|
| Free acid | | | |
| Acetone + hexane | 3 | 17.6 | 2.13 |
| Acetone + hexane + benzene | 4 | 12.8 | 2.18 |
| Methyl ester | | | |
| Methanol + $H_2O$ | 2 | 6.3 | 2.17 |
| Methanol + $H_2O$ | 2 | 3.9 | 2.21 |

[a] The specific activities for methyl cholate have been corrected on a molar basis to the free acid.

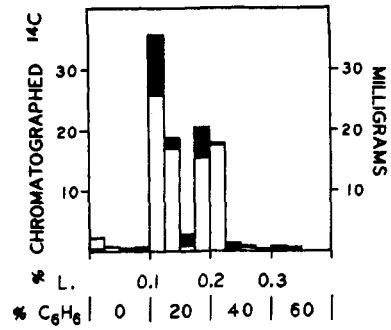



FIGURE 4: Chromatography of the residues from fractions 80-1 and 80-2 (Figure 1) after acetylation with a mixture of acetic anhydride and pyridine. The heights of the open bars from the base line to the top indicate amount of eluted solids. The heights of the solid bars from the base line to the top indicate the percentage of chromatographed $^{14}C$ eluted. The composition of the eluent (per cent $C_6H_6$) is given as the percentage of benzene in hexane. The volume of eluent is given in liters (L).

1.39% of the radioactivity in the dihydroxy fractions was present as 6β,12α-dihydroxycholanoic acid; this is equivalent to 0.74% of the chromatographed $^{14}C$ of the biliary acid fraction from rat B.

*Identification of Deoxycholic Acid.* From fractions 20-3 through 40-3 (Figure 1) which contained 23.8% of the chromatographed $^{14}C$, an aliquot ($2.56 \times 10^5$ dpm) was methylated with diazomethane, diluted with 46.8 mg of authentic methyl deoxycholate, and chromatographed on a partition column (Figure 3B). Fractions 0-2 through 20-1 were combined and the residue crystallized to constant specific activity (Table III). The remaining methyl ester was acetylated with acetic anhydride in pyridine. The product was chromatographed on a column of silica gel and the mass eluted with 20% benzene in ether was combined and crystallized from aqueous methanol to constant specific activity (Table III). Of the radioactivity in this zone, 76.9% was present in deoxycholic acid, which is equivalent to 18.3% of the chromatographed $^{14}C$.

An aliquot from the dihydroxy acid region of rat B (fractions 0-4 through 40-3) was methylated with diazomethane, diluted with authentic methyl deoxycholate, and treated similarly. From the specific activities of methyl deoxycholate and methyl 3α,12α-diacetoxycholanoate, it was calculated that 15.5% of the chromatographed $^{14}C$ was present as deoxycholic acid.

*Isolation and Identification of Cholic Acid.* Fractions 80-1 and 80-2 (rat A, Figure 1) which contained $1.82 \times 10^6$ dpm or 18.0% of the chromatographed $^{14}C$ were combined and acetylated with acetic anhydride in pyridine; the products were chromatographed on a partition column (Figure 4). The residue in fractions 20-1 and 20-2, which normally contains the triacetate of cholic acid, was combined and hydrolyzed in 5% methanolic KOH, and the material was purified by partition chromatography. The free acid thus obtained from fraction 80-2 was crystallized to a constant specific activity (Table IV). After methylation of the acid with diazomethane and chromatography of the ester on a partition column, the residue from fractions 40-3 and 40-4 was combined and crystallized from aqueous methanol. Melting point and mixture melting point determinations of the acid and the methyl ester confirmed the identity of this material. The specific activities for cholic acid and the methyl ester remained constant (Table IV). From the specific activity it was calculated that 16.5% of the chromatographed $^{14}C$ from rat A represents cholic acid. This calculation is based on the assumption that all the mass in fractions 80-1 and 80-2 (75.7 mg, Figure 1) was cholic acid.

From rat B, fractions 60-4 through 80-4 were combined, one-tenth of the mixture containing $2.52 \times 10^5$ dpm and 8.3 mg of cholic acid (Irwin *et al.*, 1944) was diluted with authentic cholic acid, and the mixture was chromatographed on a partition column. Fractions containing cholic acid were combined and acetylated with acetic anhydride in pyridine, and the products were chromatographed on a partition column. The residue in fraction 40-1, which has the characteristic chromatographic mobility of 3α,7α-diacetoxy-12α-hydroxycholanoic acid, was crystallized from a mixture of ethyl acetate and hexane. The crystalline material was then hydrolyzed in 5% methanolic KOH, purified by chromatography, and methylated with diazomethane. Specific activities were determined for 3α,7α-diacetoxy-12α-hydroxycholic acid, cholic acid, and methyl cholate as shown in Table V. Calculations from these data showed that 56.4% of the radioactivity in the fractions studied was present in cholic acid; this is equivalent to 14.2% of the chromatographed $^{14}C$. These data indicate the presence of an unidentified metabolite in these fractions, particularly in fraction 60-4 (Figure 1). Additional experiments to characterize this material are in progress.

## Discussion

Results of these experiments demonstrate a rapid and extensive metabolism of 12α-hydroxycholanoic acid to

TABLE V: Identification of Cholic Acid[a] in Bile of Rat B.

| Solvent | Crystzn | Amt (mg) | Sp Act. (dpm/mg × $10^{-3}$) |
|---|---|---|---|
| Cholic acid diacetate | | | |
| Ethyl acetate + hexane | 2 | 23.2 | 3.29 |
| Cholic acid | | | |
| Acetone + $H_2O$ | 2 | 9.1 | 2.97 |
| Methyl cholate | | | |
| Methanol + $H_2O$ | 2 | 5.3 | 2.91 |

[a] Authentic cholic acid (40.509 mg) was added to $2.52 \times 10^5$ dpm containing 8.3 mg of cholic acid to give an original specific activity of $5.16 \times 10^3$ dpm per mg. The specific activities have been corrected on a molar basis to the free acid.

TABLE VI: Molecular Rotation of Bile Acids and Derivatives.

| | $M_D$ | |
|---|---|---|
| | Calcd | Obsd |
| Methyl 12α-hydroxycholanoate | +179 | +175 |
| Methyl 12-ketocholanoate | +356 | +376 |
| 7α,12α-dihydroxycholanoic acid | +93 | +107 |
| Methyl 7-keto-12α-hydroxycholanoate | −45 | −69 |
| Methyl 7-keto-12α-acetoxycholanoate | +142 | +121 |
| 6β,12α-Dihydroxycholanoic acid | +179 | +170 |
| 6β-Hydroxy-12-ketocholanoic acid | +356 | +335 |
| 6β-Hydroxycholanoic acid | +86 | +116 |
| 6α-Hydroxycholanoic acid | −21 | |

more polar acids after intraperitoneal administration to the rat with a cannulated bile duct. Within 24 hr most of the administered $^{14}C$ was recovered in bile as 7α,12α-dihydroxycholanoic acid (26.2%), deoxycholic acid (15.5%), cholic acid (14.2%), and unchanged 12α-hydroxycholanoic acid (11.8%). The identification of 7α,12α-dihydroxycholanoic acid in those fractions containing the largest amount of excreted $^{14}C$ (fractions 0-4, 20-1, and 20-2; Figure 1) corroborates and extends our previous finding of the ability of the rat to oxygenate the cholanoic acid nucleus at position 7 in the absence of a 3α-hydroxyl group (Ray *et al.*, 1961). Whether radioactive 7α,12α-dihydroxycholanoic acid was an intermediate in the formation of cholic acid-$^{14}C$ cannot be determined from the present experiments, since deoxycholic acid-$^{14}C$ was also formed, which is effectively converted into cholic acid (Bergström *et al.*, 1958).

Since the rat is able to convert cholanoic, lithocholic, chenodeoxycholic, and deoxycholic acids into 6β-hydroxy acids, the possibility of a 6β hydroxylation of 12α-hydroxycholanoic acid was investigated. 6β,12α-Dihydroxycholanoic acid was prepared by a procedure analogous to that of Ratliff *et al.* (1961) for the preparation of 3α,6β,12α-trihydroxycholanoic acid from methyl cholate. Support for the structure of the new bile acid was obtained by unambiguous conversion to the known 6β-hydroxycholanoic acid, and the agreement between the calculated and observed values of molecular rotation, $M_D$, of the new acid, and its derivatives, 6β-hydroxy-12-ketocholanoic acid and 6β-hydroxycholanoic acid (Table VI). By isotopic dilution a small amount of this acid (0.7% maximal) was detected in fractions 0-4 through 40-3. These data suggest a very limited ability of the liver enzymes to oxygenate 12α-hydroxycholanoic in the 6β position, whereas this is so readily effected with the 3α-hydroxy derivatives (Thomas *et al.*, 1964). It is interesting to note that less than 0.6% of Ratliff's metabolite, 3α,6β-12α-trihydroxycholanoic acid (Ratliff *et al.*, 1959) was first reported (Mahowald *et al.*, 1957) in experiments comparable to these, and less than 0.6% of 3α,6β-dihydroxycholanoic acid could be present as a metabolite of cholanoic acid (Ray, 1962). In the latter case the presence of such a small amount of this metabolite may reflect a rapid hydroxylation to the muricholic acids. In the present experiments this would suggest a similar hydroxylation at position 7 of the identified 6β,12α-dihydroxycholanoic acid. Experiments to test this hypothesis are in progress.

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