Bile acids of marsupials. 2. Hepatic formation of vulpecholic acid (1α,3α,7α-trihydroxy-5β-cholan-24-oic acid) from chenodeoxycholic acid in a marsupial, *Trichosurus vulpecula* (Lesson)[1]

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Abstract Free vulpecholic acid (1α,3α,7α-trihydroxy-5β-cholan-24-oic) is the major biliary component of the Australian opossum (*Trichosurus vulpecula*), accompanied only by a few percent of its taurine conjugate. In order to exclude a microbial involvement in its formation (i.e., secondary origin) four sets of experiments were performed. It was found that *a*) the level of vulpecholic acid remained unchanged in the bile of opossums fed with neomycin and kanamycin for 7 days prior to bile collection; *b*) it also remained unchanged after long bile drainage; *c*) in opossums prepared with biliary cannula, intraportally injected [24-^{14}C]chenodeoxycholic acid was transformed to [24-^{14}C]vulpecholic acid; and *d*) in a similar experiment, the detectable transformation of [1α,2α-$^{3}H_2$]cholesterol to vulpecholic acid was observed. In experiment *c*) 28–66% of the administered radioactivity was secreted in 2 h in the form of free biliary vulpecholic and chenodeoxycholic acids. Only a trace amount of the corresponding taurine conjugates (~0.4%) was formed. Moreover, rapidly declining specific radioactivity of the unconjugated chenodeoxycholic acid indicated its probable participation in the native formation of vulpecholic acid. —**St. Pyrek, J., S. P. Lee, L. Thomsen, C. Tasman-Jones, and B. Leydon.** Bile acids of marsupials. 2. Hepatic formation of vulpecholic acid (1α,3α,7α-trihydroxy-5β-cholan-24-oic acid) from chenodeoxycholic acid in a marsupial, *Trichosurus vulpecula* (Lesson). *J. Lipid Res.* 1991. **32:** 1417–1427.

Supplementary key words Australian opossum • dehydro-vulpecholic acid • cholesterol

Vulpecholic acid (VCA, 1α,3α,7α-trihydroxy-5β-cholan-24-oic acid, **Fig. 1**), the principal component of the bile of the Australian opossum (*Trichosurus vulpecula*), is the first example of 1α-hydroxylated bile acid (BA) (1). Only 1β-hydroxylated BA are known as minor components of human urine of the adult (2–6) and the newborn (7–10), meconium (11–13), bile of certain reptiles (14), and products of microbial hydroxylations (15).[3] The prevalent presence of VCA in this bile as a free BA is the marked exception for a mammal. Equally unexpected is the complete absence of cholesterol and phospholipids (1). These unique characteristics of *T. vulpecula* inspire further investigation and the present report addresses verification of the hepatic synthesis of VCA.

EXPERIMENTAL[4]

Animal experiments

Opossums trapped in the bush near Auckland, New Zealand, were kept on a vegetarian diet as described earlier (1). Before bile collection, animals were fasted for

Abbreviations: BA, bile acid; CDCA, chenodeoxycholic, 3α,7α-dihydroxy-5β-cholan-24-oic acid; allo-CDCA, allo-chenodeoxycholic, 3α,7α-dihydroxy-5α-cholan-24-oic acid; DEAP-LIPIDEX, diethylaminopropyl lipophilic Sephadex; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; CGH, choloylglycyl hydrolase; HPLC, high pressure liquid chromatography; MTBE, methyl-*tert*-butyl ether; NMR, nuclear magnetic resonance; *p*-BPA, *p*-bromophenacyl ester; RP-MPLC, reverse phase-medium pressure liquid chromatography; TLC; thin-layer chromatography; VCA, vulpecholic, 1α,3α,7α-trihydroxy-5β-cholan-24-oic acid.

[1]For part 1, see ref. 1.

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[3]Identifications of 1β-hydroxylated BA, with the exception of the urine component (4) and the microbial hydroxylation product (15) characterized by NMR, have been performed solely by GC-MS using the diagnostic fragmentation of trimethylsilyl ethers leading to the ion at *m/z* 217. This, however, may not be sufficient for the definitive identification because this fragment ion is present both in 1α and 1β hydroxylated BA. The feasibility of the chromatographic resolution of 1-epimeric pairs of differently substituted BA, crucial for this distinction, is not known.

[4]Full description of experimental and analytical procedures is given in ref. 1.

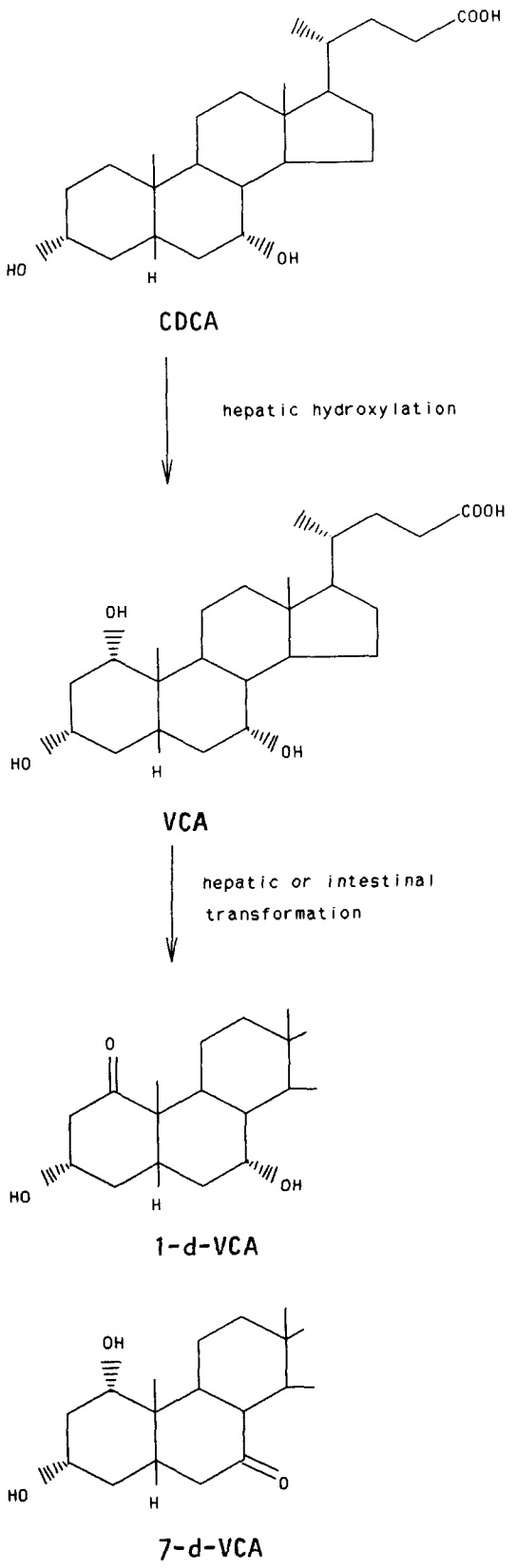


Fig. 1. Chenodeoxycholic (CDCA), vulpecholic (VCA), 1-dehydro-vulpecholic (1-d-VCA), and 7-dehydro-vulpecholic acids (7-d-VCA).

12 h. Animals were anesthetized with urethane prior to laparotomy and cannulation of the common bile duct. Because these animals are not amenable to prolonged restraint after a surgical procedures, only a short time of bile collection was usually possible.

Thin-layer chromatography

Three solvent systems were used for TLC analysis and preparative separation of BA: A, benzene–acetone 1:1 (v/v) acidified with a few drops of acetic acid; B, benzene–acetone 2:1; and C, chloroform–isopropanol–acetic acid–water 48:48:2.4:0.6. Plates (Whatman Inc., Clifton, NJ; HPTLC-Fertigplatten, EM Science, Darmstadt, Germany) were first developed overnight to remove impurities and to equilibrate silica gel with the acidic solvent, and air dried. In order to obtain better resolution selected plates were developed at 0°C. Autoradiograms were obtained using X-ray film (Eastman-Kodak, Rochester, NY). Two exposures, one for several days and the second for at least 1 month, were performed for each analytical plate. A short overnight exposure was usually sufficient for preparative plates and was performed at −70°C in order to avoid decomposition of compounds on silica gel; this was followed by spraying with water for the detection of all BA bands.

Ion exchange chromatography

The column of DEAP-LIPIDEX (Packard, Downers Grove, IL, 1.5 × 23 cm) was regenerated by the successive washing with 0.5 N potassium phosphate in 72% ethanol followed by 72% ethanol at about 50 PSI (1, 16). Solutions, desalted on a small column of either Amberlyst A-15/H^+ (Aldrich Chemical Company, Milwaukee, WI) or "SO_3H" ion exchanger cartridge (Analytichem International, Cambridge, England), were directly applied through the MPLC pump in 72% ethanol. The column was eluted with 72% ethanol (150 ml), 0.1 N acetic acid (150 ml), and a linear gradient prepared from 0.1 N acetic acid (450 ml) and 0.1 N ammonium acetate (pH 9.5, 450 ml) all in 72% ethanol (1). Fractions were analyzed by scintillation counting and TLC-autoradiography in solvents A (fractions 1–50) and C (fractions 40–120).

Medium pressure–reverse phase chromatography

Two C_8 silica gel columns connected in series (RP-8, LOBAR, EM Science, Darmstadt, Germany, 1 × 24 cm) were used for the separation of free BA. Regeneration was performed with 100% ethanol followed by 36% ethanol. Samples dissolved in 36% ethanol were applied through the MPLC pump and eluted with 36% ethanol (150 ml) followed by the linear gradient prepared from 36% (450 ml) and 72% ethanol (450 ml). All these solvents were acidified with acetic acid at the concentration of 0.01 M. About 120 fractions were collected and analyzed as above.

High pressure liquid chromatography

An HPLC system, composed of μLC-500 pump (ISCO, Lincoln, NE), loop injectors (1 and 10 μl), 3 μ C_{18} column (25 × 0.21 cm, and 5 × 0.5 cm), and μ-LC-10 UV detector, set at 257 nm, was applied for the separation of *p*-bromophenacyl ester (*p*-BPA) of BA. For elution 85% methanol was used with the flow rate of 0.2 ml/min, for the first, and 0.5 ml/min, for the second column and, if better resolution was required, 80% methanol was used.

Derivatization of free BA was performed in dry acetonitrile at room temperature with an excess of *p*-bromophenacyl bromide (freshly recrystallized from methanol, λ_{max} 260 nm log ε = 4.15, in methanol) and triethylamine. This simple overnight reaction was quantitative and was preferred to the more forceful method involving crown ethers (17) because the latter led to the formation of additional UV absorbing compounds. In order to lower chromatographic interferences, especially in the case of reactions performed for very small amounts of BA with a large excess of reagents, the additional purification by preparative TLC (solvent C, detection by the overnight autoradiography) was performed before HPLC analysis.

Determination of specific radioactivity

Specific radioactivity of crystalline samples of the biosynthesized BA was determined gravimetrically (microbalance) taken in the amount permitting at least 2% counting accuracy (0.3–0.5 mg). Samples, dissolved in a few drops of methanol, were counted in 5 ml of a liquid scintillator. Specific actvity of CDCA was determined by HPLC of *p*-BPA esters. The UV response at 257 nm was calibrated with VCA of the specific activity determined gravimetrically (32.0 dpm/μg, corresponding to 0.00614 μCi/μmol of the VCA monohydrate). Seven injections of this standard resulted in R = $2.99 \times 10^{-4} \pm 1.6 \times 10^{-5}$ (dpm/chromatogram area units) representing the ratio of the total radioactivity in the chromatographic peak and the integral area measured at 0.1 absorption units, 0.5 cm path. The factor of 20.6 ± 1.1, obtained from this measurement, was used in the calculation of specific activity expressed in μCi/μmol.

p-*Bromophenacyl ester (*p-*BPA) of CDCA,* non-crystalline, purified by preparative TLC; λ_{max} 254 nm log ε = 4.22 in methanol; NMR: δ ($CDCl_3$, 300 MHz) 0.675 s (Me-18), 0.909 s (Me-19), 0.960 d (J = 6.3 Hz, Me-21), 2.3–2.6 m (CH_2-23), 3.47 m (3β-H), 3.85 bq (J = 2.7, H-7β), 5.282 s (O-CH_2CO-), 7.64 d and 7.78 d (J = 8.4 Hz, p-C_6H_4-Br); MS: *m/z* (70 eV, % rel. int.) 572, 570 (M-H_2O, 0.5), 557, 555 (M-H_2O-Me, 1), 554, 552 (M-2×H_2O, 3), 539, 537 (M-2×H_2O-Me, 3), 500, 498 (M-Cl..3-H_2O, 0.5), 391, 389 (M-AB-H, 0.5), 373 (M-$CH_2COC_6H_4Br$-H_2O, 10), 355 (M-$CH_2COC_6H_4Br$-2×H_2O, 50), 339 (M-$OCH_2COC_6H_4Br$-2×H_2O, 18), 289 (10), 288 (7), 273 (M-SC-H_2O, 6), 271 (7), 264 (M-SC-C16..17, 12), 255 (M-SC-2×H_2O, 19), 246 (M-SC-C16..17-H_2O, 18), 231 (M-SC-C16..17-H_2O-Me, 8), 229 (19), 228 (M-SC-C16..17-2×H_2O, 27), 213 (M-SC-C16..17-2×H_2O-Me, 28), 185, 183 (COC_6H_4Br, 100).

p-*Bromophenacyl ester (*p-*BPA) of VCA,* non-crystalline, purified by preparative TLC; λ_{max} 254 nm log ε = 4.23 in methanol; NMR: δ ($CDCl_3$, 300 MHz) 0.686 s (Me-18), 0.954 d (J = 6.6 Hz, Me-21), 1.117 s (Me-19), 2.3–2.6 m (H_2-23), 3.30 bd (H-1β), 3.52 br (H-3β), 3.86 bs (H-7β), 5.28 s (O-CH_2CO-), 7.64 d and 7.78 d (J = 8.1 Hz, p-C_6H_4-Br); MS: *m/z* (EI, 70 eV, % rel. int.) 588, 586 (M-H_2O, 1.4), 570, 568 (M-2×H_2O, 2.6), 555, 553 (M-2×H_2O-Me, 0.6), 552, 550 (M-3×H_2O, 1.4), 537, 535 (M-3×H_2O-Me, 0.6), 500, 498 (M-Cl..4-H_2O, 2), 485, 483 (M-Cl..4-H_2O-Me, 0.5), 407, 405 (M-AB-H, 2), 389 (M-$CH_2COC_6H_4Br$-H_2O, 31), 371 (M-$CH_2COC_6H_4Br$-2×H_2O, 16), 355 (M-$OCH_2COC_6H_4Br$-2×H_2O, 7), 353 (M-$CH_2COC_6H_4Br$-3×H_2O, 25), 337 (M-$OCH_2COC_6H_4Br$-3×H_2O, 5), 302 (13), 301 (14), 287 (8), 271 (M-SC-2×H_2O, 12), 262 (M-SC-C16..17-H_2O, 15), 253 (M-3×H_2O, 7), 244 (M-SC-C16..17-2×H_2O, 18), 211 (M-SC-C16..17-3×H_2O-Me, 41), 209 (30), 185, 183 (COC_6H_4Br, 100). MS (isobutane, +CI) 607, 605 (MH^+, 3), 589, 587 (MH-H_2O, 17), 571, 569 (MH-2×H_2O, 35), 553, 551 (M-3×H_2O, 15), 447, 445 (4), 415 (6), 409, 407 (5), 397, 395 (~20), 391 (MH-$HOCH_2CO$-C_6H_4Br, 48), 373 (ion 391-H_2O, 100), 355 (ion 391-2×H_2O, 48), 337 (ion 391-3×H_2O, 5), 217, 215 (protonated $HOCH_2CO$-C_6H_4Br, 90), 201, 199 (protonated $CH_3COC_6H_4Br$, 50).

Purification of radioactive chenodeoxycholic acid

Commercial [24-^{14}C]CDCA (Amersham, England and New England Nuclear, Boston, MA) was purified by RP-MPLC. The first sample (20.9 μCi, quoted as 59.8 mCi/mmol) yielded upon this separation 18.5 μCi, of CDCA. The purified [24-^{14}C]CDCA, homogeneous according to RP-MPLC and TLC-autoradiography (solvent A), was used for the injection into animals #21 and #22. The second sample of [24-^{14}C]CDCA (59.5 μCi, quoted as 50 μCi/μmol) yielded 57.3 μCi of CDCA, homogeneous by TLC-autoradiography, with the specific radioactivity measured as 40.7 μCi/μmol (1.62×10^5 DPM/μg). This acid was utilized for experiments performed with animals #42 as well as two animals #45, and #46 described separately.

Hydrolysis with choloylglycyl hydrolase

Enzymatic hydrolysis was performed in 0.025 M phosphate buffer, pH 5.6, containing 0.1% of 2-mercaptoethanol and ~1 mg/5 ml of the enzyme (CGH, 160 units/mg Sigma, St. Louis, MO). The reaction mixture was incubated at 38–40°C for 1–3 days under nitrogen and

stopped by filtration through a C_{18} cartridge prewashed with alcohol and water. After washing with water (10 ml), free BA precipitated in the reaction and absorbed on the cartridge were eluted with ethanol (10 ml).

Alkaline hydrolysis of conjugated BA

Bile samples obtained from animals #2–5 and control animals #1, 6–11 were hydrolyzed in aqueous potassium hydroxide (1 ml, 5%) at 120°C for 12 h. Subsequently the solution was diluted tenfold with water and applied to a C_{18} cartridge (0.5 g, Analytichem International, Cambridge, England) and washed additionally with water (20 ml). This efficiently removed trace silicates formed by the partial dissolution of the glass test tube. BA salts were eluted with methanol (5 ml) and after evaporation of methanol converted to free acids by extraction with ethyl acetate from 1% sulfuric acid (2 ml). Extracts were dried with sodium sulfate, evaporated with nitrogen, redissolved in methanol, and methylated with diazomethane in ethyl ether. Subsequent acetylation, performed at 0°C with the mixture of acetic acid–acetic anhydride–perchloric acid 7:3:0.01 (v/v/v) afforded methyl ester acetates isolated after dilution with water and extraction with hexane.

RESULTS

Formation of VCA in animals fed with antibiotics and animals subjected to bile drainage

Opossums #2–5: Two animals (#2, male, 7.8 kg and #3, female, 5.6 kg) were fed with kanamycin and neomycin (0.34 g/kg body weight/day of each antibiotic) for 7 days before the collection of gallbladder bile in the amount of 2.25 ml (for animal #2) and 2.50 ml (for animal #3). Two other animals (#4, female, 6.2 kg, and #5, male, 7.2 kg) were subjected to cholecystectomy and prepared with biliary fistula introduced to the common bile duct. The surgical incisions were closed and the animals were placed in body restraint cages with free access to food and water. Bile was drained for 24 h before the final collection of samples in the amount of 10.25 ml (for animal #4) and 8.50 ml (for animal #5).

Samples of the lyophilized bile were separated by preparative TLC (solvent C) into two bands. The less polar one corresponded to the unconjugated VCA. As determined gravimetrically, it was the principal component of all samples investigated and constituted, respectively, 66, 60, 64, and 70% of the bile solid. The second band, from the above TLC separation, remained close to the origin and corresponded to taurine-conjugated BA. This material was submitted to alkaline hydrolysis to give free BA. BA composition was quantified by the capillary GC of methyl ester acetate derivatives using n-C_{30} hydrocarbon as the internal standard (DB-17 capillary, J&W, Folsom, CA, 30 m, 0.25 mm ID, 0.1 μm at 290°C N_2, 15 PSI). The amount of VCA found in the taurine fraction in four of these animals was 0.3, 8.2, 0.5, and 0.9% of the bile solid, respectively. The amount of CDCA and allo-CDCA[5] was 1.6, 2.7, 2.3, and 3.2%, respectively. These two dihydroxylated acids were not separated as methyl ester-diacetate derivatives under the above conditions.

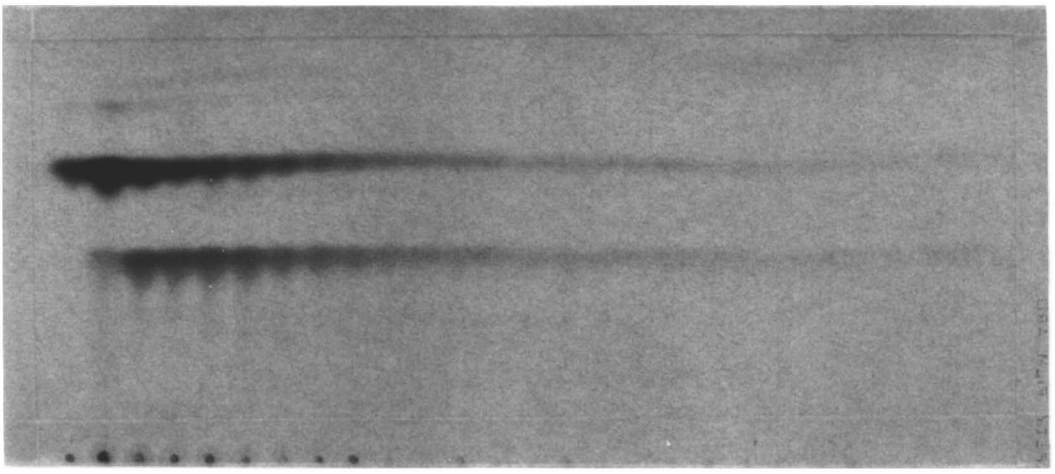
Gallbladder bile of seven control animals (#1, 6–11) was subjected to the same procedure. Animals #1, 6, and 7 were analyzed for the amount of free VCA which was found as 62.0, 65.2, and 69.0% of the bile solid, respectively. Conjugated BA were measured for all seven animals and VCA was found as 0.3, 0.6, 0.6, 0.4, 2.7, 0.7, and 1.6%, respectively, whereas CDCA, and allo-CDCA were found in the cumulative amounts of 0.7, 2.7, 3.0, 0.9, 6.0, 6.0, and 6.7, respectively.

Transformation of chenodeoxycholic acid into vulpecholic acid

Opossums #10, 21, 22, and 42: Four opossums (#10, female, 1.9 kg, #21, female, 4.8 kg, #22, male, 5.6 kg, and #42, male, weight not recorded) were cannulated and their bile was drained for 30 min prior to the injection of the solution of [24-^{14}C]CDCA into the portal vein. Bile samples were collected in either 15- or 30-min time intervals for 2–6 h. Each sample, frozen in liquid nitrogen, was lyophilized separately and sealed under nitrogen. Before analysis the bile solid was dissolved in 72% ethanol. Only a negligible amount of material remained undissolved or precipitated upon storage of these solutions.

Animal #10 was injected with 10 μCi of [24-^{14}C]CDCA and the hepatic bile was collected for 7 h in 15-min intervals. Close to 58% of the injected dose was recovered in the bile with the maximum output in the first half hour (**Fig. 2**). According to autoradiography (solvent A, two developments), the radioactivity in the free CDCA (R_f 0.53) dominated between 15 and 30 min. The radioactivity present in the major spot of VCA (R_f 0.30) appeared in 30 min and dominated between 45 and 75 min. Only a small amount of radioactivity was detected in keto-derivatives[5] of VCA (R_f 0.45) and conjugated BA (solvent C). In addition, but only upon very long autoradiography, three faint spots of nonpolar radioactive compounds were detected and corresponded according to their R_f values to the unmetabolized impurities present in the original sample of radioactive CDCA used for this injection. Apart from changes in the distribution of radioactivity, no ap-

[5]Identification of other BA present in the bile of *T. vulpecula* and of further incorporation experiments is described separately. Minor BA include allo-CDCA, 1-oxo, and 7-oxo dehydro-VCA, whereas nor-VCA, nor- and bisnor-CDCA and allo-CDCA are present as trace BA. Lithocholic acid and its isomers are the major fecal BA, whereas a trace of lithocholic acid and 7-deoxy-VCA occurs in the bile as 3-O-β-glucuronides.



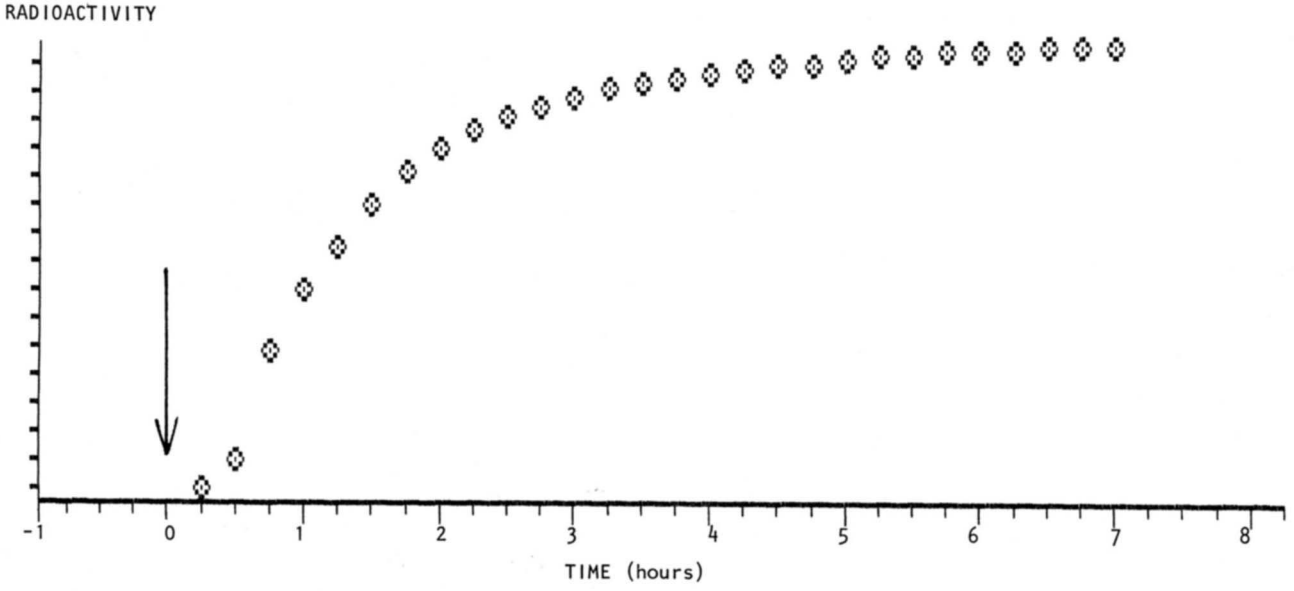


Fig. 2. Bottom: Recovery of the ^{14}C radioactivity in the bile of opossum #10 injected with [24-^{14}C]CDCA; about 73% of injected dose was secreted in 7 h. Top: Autoradiogram of the corresponding bile fractions obtained after TLC in solvent A.

parent difference in the composition of fractions 0–27 was noted by TLC analysis.

Bile samples 1–27 (366 mg of the solid material) were combined and dissolved in 72% ethanol (28.5 ml, containing 5.77 μCi of the ^{14}C radioactivity). Triple extraction with hexane (24 ml total volume) removed only a trace (0.17%) of this radioactivity. The separation on DEAP-LIPIDEX produced neutral components (3.1% of the recovered radioactivity, fractions 3–6), free BA (95%, 220 mg, fractions 27–40), taurine-conjugated BA (0.7%, fractions 52–64), and BA glucuronides[6] (1.2%, fractions 70–88). Free BA were separated into individual components by C_8 RP-MPLC to give the following components.

VCA, the major component eluted in fractions 36–45 (31.9% of the radioactivity applied to this column), was recrystallized from 40% ethanol to give two crops of the homogeneous acid (TLC, solvent A). The specific activity was comparable to that of the residual mother liquor (**Table 1**). Two additional recrystallizations of the second crop of VCA from acetone–water raised the melting point but its specific radioactivity remained unchanged. The first crop of this VCA was subsequently administered to animals #43 and #44, described separately.

VCA ethyl ester, eluted in fractions 67–71 as a trace component, was hydrolyzed at room temperature with 1 M NaOH. Specific activity of the free acid, transformed to its *p*-BPA derivative, was determined by HPLC as

[6]Two types of BA glucuronides, hydroxyl-attached and carboxyl-attached, are well separable by the DEAP-LIPIDEX ion exchange chromatography. This separation, due to the presence of two and one carboxyl groups, respectively, ought to be underlined in view of the recent discussion of the methodology in BA analysis (20).

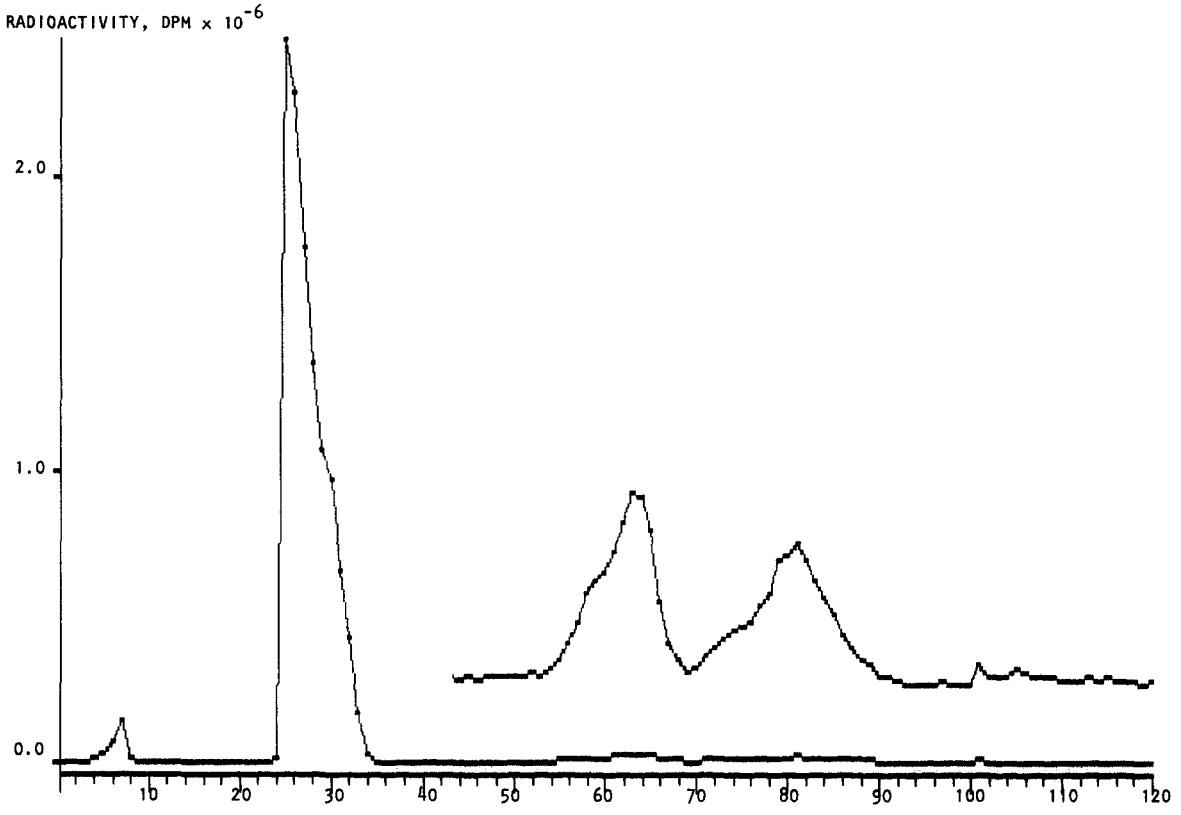


Fig. 3. Radioactivity profile of DEAP-LIPIDEX, ion exchange separation of radioactive bile components obtained from the combined bile of opossum #21.

0.00447 μCi/μmol, equivalent to 23.3 dpm/μg, i.e., close to that of the major fraction of VCA. This confirmed the operation of a partial esterification during work-up and chromatography in ethanol-containing solvents rather than its native presence as ethyl ester.

7-Dehydro-VCA (1α,3α-dihydroxy-7-oxo-5β-cholan-24-oic acid, fractions 24–34, R_f 0.40, 15.6 mg) containing 1.5% of the radioactivity applied to this column (0.66% of the injected dose of CDCA) was recrystallized from diluted acetone (Table 1).

CDCA (fractions 78–84, R_f 0.40, 60.8% of the radioactivity applied to the column) according to HPLC of *p*-BPA derivative, had the high specific activity of about 14 μCi/μmol. This corresponded to about 0.10 mg of CDCA present in this fraction together with about 0.05 mg of the non-radioactive allo-CDCA.

Several minor BA, obtained from this C_8 RP-MPLC separation, included the non-radioactive 1-dehydro-VCA (3α,7α-dihydroxy-1-oxo-5β-cholan-24-oic acid, fractions 46–55, R_f 0.42, 3.2 mg).

Animal #21 was injected with 8.36 μCi [24-^{14}C]CDCA and the hepatic bile was collected in four 30-min fractions of 1.50, 0.95, 0.70, and 0.70 ml, respectively. These fractions contained 79, 44, 33, and 48 mg of the solid material and 44.1, 8.2, 2.8, and 1.6% of the injected radioactivity, respectively. Apart from a very small amount of the radioactivity in conjugated BA(s) and 7-dehydro-VCA, the analysis by TLC–autoradiography showed only two significant radioactive spots of free CDCA and VCA with the approximate ratios of 84:16, 53:47, 61:39, and 65:35, respectively. Hexane extraction of combined samples dissolved in aqueous alcohol removed only 0.04% of the total radioactivity from this solution. Subsequent DEAP-LIPIDEX chromatography produced neutral components (2.3% of the original radioactivity, fractions 4–8), free BA (96.5% equivalent to 4.55 μCi of ^{14}C, ~140 mg, fractions 24–34), taurine-conjugated BA (0.46%, fractions 53–68), and bile acid glucuronides (0.46%, fractions 70–90) (**Fig. 3**).

Free BA were separated by C_8 RP-MPLC to give **VCA** recrystallized from aqueous ethanol (fractions 32–39, 29.7% of the radioactivity applied to this separation, **Fig. 4**). Four successive recrystallizations of the second crop, showing relatively constant mp and specific activity, confirmed the radiochemical purity of this VCA (Table 1). The first crop of VCA was used for the injection to opossum #47, described separatively.

VCA ethyl ester was eluted in fractions 56–66 and contained only 0.44% of the radioactivity applied to the

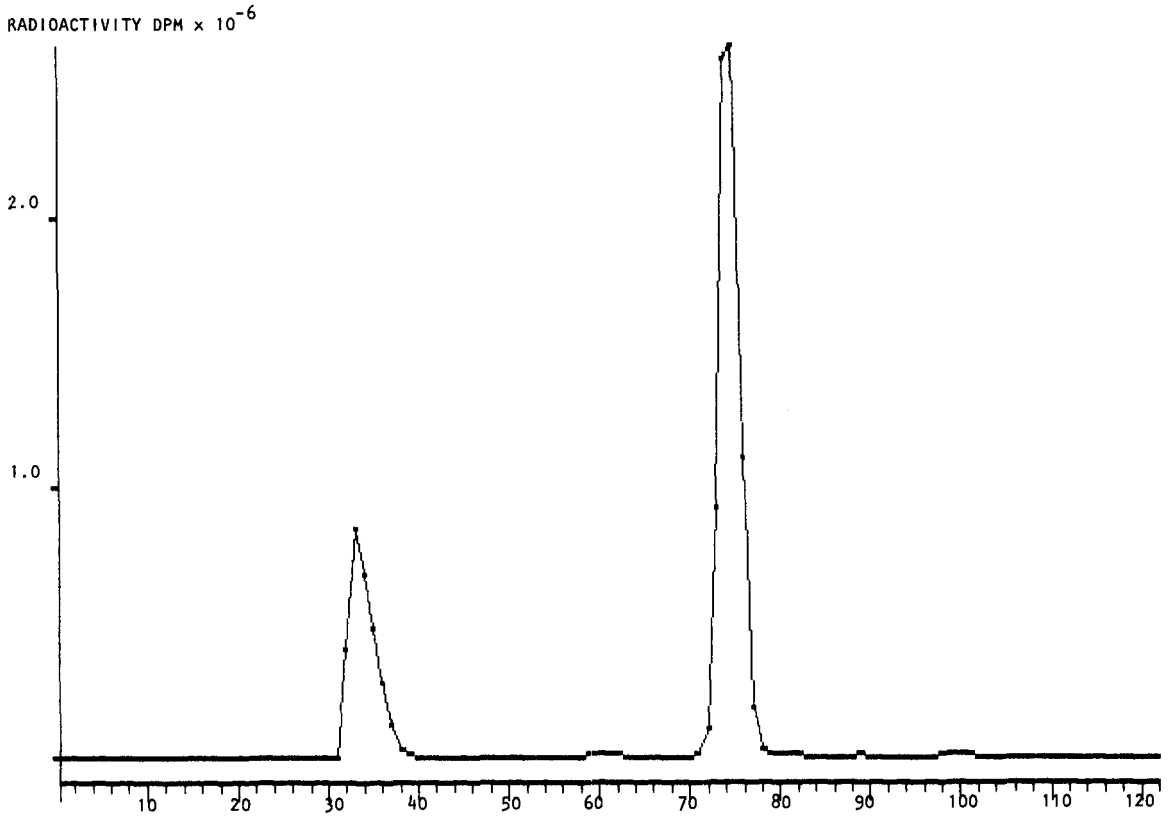


Fig. 4. Radioactivity profile of the C_8 silica gel reverse phase separation of unconjugated bile acids separated from the bile of opossum #21.

column. Its specific radioactivity, determined by HPLC of *p*-BPA, was 0.0057 μCi/μmol, corresponding to 30 dpm/μg, i.e., close to that of the major fraction of VCA (compare the comment above).

Part of CDCA (fractions 71–80, 68.7% of the recovered radioactivity) was separated by preparative TLC (solvent A) to give three bands detected by spraying with water and the 48-h autoradiography. Almost all radioactivity migrated with the band of CDCA (R_f 0.51, 2.05 μCi). Two more polar bands (R_f 0.32 and 0.12) were apparently not radioactive. The specific activity of CDCA was determined by HPLC of *p*-BPA derivative (Table 1). The nonradioactive allo-CDCA (~0.05 mg) was present in the same fraction.

Other BA obtained from this C_8 RP-MPLC separation included 7-dehydro-VCA (fractions 25–30, 9.5 mg, 0.11% of the radioactivity applied to this separation) recrystallized from diluted ethanol (Table 1).

Taurine-conjugated BA contained only 0.024 μCi, i.e., 0.46% of the recovered radioactivity injected in animal #21. Amongst three components (TLC in solvent B: R_f 0.03, 0.06, and 0.09) the major (R_f 0.09) corresponded to the standard of tauro-CDCA. Two successive treatments with CGH, 1 day each, and C_{18} solid extraction recovered 11.1 mg (0.022 μCi) of free BA. Preparative TLC (solvent A, followed by 48 h autoradiography) gave a mixture of CDCA and allo-CDCA of very low specific activity (R_f 0.58, 6.5 mg, 0.0035 μCi), and VCA (R_f 0.38, ~1.2 mg, 0.0059 μCi) along with other minor components.

Opossum #22, injected with 8.36 μCi [24-^{14}C]CDCA, produced four samples of hepatic bile in the amounts of 0.40, 0.40, 0.25, and 0.25 ml, respectively, in 2 h. The solid material obtained in the amounts of 24, 17, 13, and 13 mg, contained only 14.8, 7.7, 3.3, and 2.2% of the injected radioactivity, respectively. Fractionation, similar to that described for animal #21, was applied to the combined bile and resulted in the nonpolar fraction of the hexane extract (0.24% of the recovered radioactivity), neutral components (2.3%, fractions 6–9), free BA (96%, fractions 22–33), taurine-conjugated BA (~0.4%, fractions 50–60, and BA glucuronides (~0.5%, fractions 61–90). Hydrolysis of the taurine fraction with CGH produced only 0.0092 μCi of free BA with composition similar to that of animal #21.

VCA (fractions 30–34 of C_8 RP-MPLC chromatography) was recrystallized from aqueous ethanol (Table 1). This acid (3.2 mg) was transformed to its methyl ester triacetate, identical by MS and NMR with the sample prepared from opossum #1 (1), and was used as the reference for the autoradiography of the C_{23} nor-bromide (see below)

TABLE 1. Transformation of radioactive chenodeoxycholic acid in opossums #10, 21, 22, and 42

Animal	Amount	Melting Point	Specific Activity	
			μCi/μmol	dpm/μg[a]
	mg	°C		
Opossum #10				
VCA Kl, lx	140.0	147–150	0.00410	21.4
K2, lx[b]	30.0[b]	153–154	0.00428	22.3
ML	8.6		0.00407	21.2
K2, 2x		155–158	0.00422	22.0
K2, 3x		157–158	0.00420	21.9
CDCA	0.1[c]		14[d]	
7-d-VCA	10.8	233–234	0.00247	13.5
Opossum #21				
VCA Kl, lx	90.5	152–155	0.00631	32.9
K2, lx[b]	6.6[b]	152–154	0.00616	32.1
K2, 2x		153–155	0.00626	32.6
K2, 3x		152–156	0.00620	32.3
K2, 4x		153–156	0.00614	32.0
CDCA	0.33[c]		3.72[d]	
7-d-VCA	8.3	233–234	0.00013	0.7
Opossum #22				
VCA Kl, lx	30.0	156–159	0.0129	67.0
7-d-VCA	3.6	233–236	0.00051	2.8
Opossum #42, samples 1–4				
1 VCA Kl, lx	37.0	154–155	0.00873	45.5
1 VCA K2, lx	6.3	151–155	0.00856	44.7
1 CDCA	2.67[c]		21.2[d]	
2 VCA Kl, lx	51.1	150–153	0.00927	48.3
2 CDCA	1.34[c]		11.3[d]	
3 VCA Kl, lx	39.7	151–154	0.00349	18.2
3 CDCA	0.90[c]		5.2[d]	
4 VCA Kl, lx	19.4	154–156	0.00274	14.3
4 CDCA	0.38[c]		3.5[d]	

[a]Gravimetric determination.
[b]Second crop (K) of VCA and its successive recrystallizations (x).
[c]Amount determined from specific radioactivity.
[d]HPLC determination.

prepared from the same sample of VCA. 7-Dehydro-VCA (3.6 mg, fractions 25–28) was recrystallized from diluted acetone (Table 1).

Decarboxylation of VCA: The above VCA (26.5 mg) was acetylated in acetic acid, acetic anhydride, and perchloric acid (3.0, 2.0, and 0.010 ml, respectively) in an ice bath for 45 min. Water (2 ml) was added and the homogeneous solution was left overnight at room temperature to hydrolyze the mixed acetic acid-VCA anhydride. Subsequently, after addition of water (15 ml), VCA triacetate was extracted four times with MTBE (10 ml total). This homogeneous triacetate (TLC) was decarboxylated with $HgO\text{-}Br_2$ in carbon tetrachloride as described before (18) to give C_{23} nor-bromide (1α,3α,7α-triacetoxy-23-bromo-5β-norcholane) purified by the two successive TLC (0.64 mg, three developments in solvent G). This bromide was identical by NMR, TLC, and capillary GC (30 m DB-5, 0.25 mm, 0.1 μm, 280°C) with the compound prepared from the non-radioactive VCA. According to the long autoradiographic comparison with radioactive VCA methyl ester triacetate obtained from the parent VCA, this bromide was completely devoid of the original radioactivity.

When this decarboxylation was performed with mg amounts of hydroxyl-protected BA it constantly produced low yield of the product; yields were much better for larger-scale reactions. NMR: δ ($CDCl_3$, 300 MHz) 0.679 s (Me-18), 0.953 d (J=6.3 Hz, Me-21), 0.992 s (Me-19), 2.027 s, 2.044 s, and 2.050 s (3×AcO), 3.30–3.55 m (CH_2-23), 4.580 dd (J=3.9 and 12.6 Hz, H-1β), 4.756 m ("septet", H-3β), and 4.863 q (J= ~2 Hz, H-7β); MS: *m/z* (15 eV, % rel. int.; HR-MS data obtained at 70 eV with the resolving power of 10,000: expected composition and error) 526, 528 (1, M-ketene), 508, 510 (8, M-AcOH; $C_{27}H_{41}O_4Br$ −5.7 and −5.3 ppm), 466, 468 (4, M-AcOH-ketene; $C_{25}H_{39}O_3Br$, −13 and −4.6 ppm), 448, 450 (100, M-2×AcOH; $C_{25}H_{37}O_2Br$, −3.1 and −3.0 ppm), 433, 435 (10, M-AcOH-Me; $C_{24}H_{34}O_2Br$, −1.9 and +0.2 ppm), 406, 408 (9, M-2×AcOH-ketene; $C_{23}H_{35}OBr$ +8 and +0.5 ppm), 388, 390 (57, M-3×AcOH; $C_{23}H_{33}Br$, −0.9 and +0.6 ppm), 373 (17, M-3×AcOH-Me; $C_{22}H_{30}Br$, −4.2 and −1.6 ppm), 347, 349 (25, M-AcOH-C1..3-H; $C_{20}H_{28}Br$, −0.3 and −0.2 ppm), 335–339 (12–20), 313 (10, M-2×AcOH-SC; $C_{21}H_{29}O_2$, −3.1 ppm) 253 (23, M-3×AcOH-SC; $C_{19}H_{25}$, −1.6 ppm), 226 (53, M-

3×AcOH-SC-C16..17; $C_{17}H_{22}$, +1.5 ppm), 211 (41, "226"-Me/M-3×AcOH-SC-C15..17-H, $C_{16}H_{19}$, −1.3 ppm).

Opossum #42 was injected with 20.06 μCi of [24-^{14}C]CDCA and secreted in the bile, respectively, 43.2, 15.9, 5.1, and 1.8% of the radioactivity in four samples with volumes of 2.3, 2.3, 1.0, and 0.4 ml collected in 30-min periods. This corresponded to a total of 13.21 μCi (65.8%). TLC examination in solvent A showed, apart from conjugated BA remaining at the origin, VCA (R_f 0.22), CDCA and allo-CDCA (R_f 0.37), and several trace components (R_f 0.33, 0.40, 0.54, and 0.57). On the same plate R_f values for standard BA were: cholic acid, 0.07; hyocholic acid, 0.17; hyodeoxycholic acid, 0.30; deoxycholic acid, 0.36; chenodeoxycholic acid, 0.37; ursodeoxycholic acid, 0.43; lithocholic acid, 0.67; and cholesterol, 0.77.

Only a small amount of radioactivity was detected in conjugated BA and VCA ethyl ester (R_f 0.57) while the major amount was associated with CDCA and VCA. These four radioactive bile samples, each separately, were submitted to the sequential DEAP-LIPIDEX and C_8 RP-MPLC separation. In all four cases free BA constituted ~96% of the radioactivity applied to DEAP-LIPIDEX. Purified VCA was recrystallized from diluted ethanol and the fraction of CDCA was transformed into the *p*-BPA derivative, purified by TLC and analyzed by HPLC (Table 1).

Transformation of [1α,2α-$^3H_2$]cholesterol to vulpecholic acid

Male opossum #11 (1.8 kg) was prepared with a biliary cannula and injected intravenously with [1α,2α-T_2]cholesterol (10 μCi) dissolved in ethanol (50 μl) and the opossum's plasma (5 ml). Bile was collected for 7 h in 15-min intervals. The combined bile, containing 0.595 μCi ^{3}H (5.95% of the dose), was passed through Amberlyst 15 H^+ in 72% ethanol (4.0 ml) and extracted four times with hexane (21 ml total) which removed only 0.012 μCi. Subsequent DEAP-LIPIDEX separation produced neutral compounds in fractions 3–6 (16% of the applied radioactivity), free BA in fractions 26–40 (32%), taurine-conjugated BA in fractions 60–71 (29%), and BA glucuronides in fractions 72–100 (15%). Free BA were spiked with a very small amount of [24-^{14}C]CDCA and [24-^{14}C]VCA and separated by RP-MPLC. VCA, present in fractions 37–62, contained only a small part of the tritium radioactivity. This acid was subjected to the multiple crystallizations from methanol-water and acetone-water (**Table 2**).

DISCUSSION

The primary character of VCA was assumed at the moment of its structure determination, mostly because of the presence of the 7-hydroxyl group (1). The present study, performed primarily in order to exclude a microbial involvement in its formation, fully confirmed this assumption, clearly indicating the hepatic synthesis of VCA observed in four different sets of experiments. Thus, both free and conjugated VCA were present at unchanged levels, when compared to control animals, in the bile obtained from opossums fed antibiotics and opossums subjected to prolonged bile drainage. The first treatment was expected either to suppress or to eliminate intestinal flora while the second was expected to deplete secondary BA from the newly secreted hepatic bile. If VCA was a secon-

TABLE 2. In vivo transformation of [1α,2α-$^3H_2$]cholesterol to VCA in opossum #11; recrystallization of VCA

	Amount[a]	Melting Point	^{3}H	^{14}C[b]	Counted[c]
	mg	*°C*	*dpm/mg*		*mg*
1x	124.6	nd	nd	nd	nd
2x	116.3	156–157	46.0	15.3	5.85
3x	109.0	~155	42.2	15.8	4.27
4x	100.0	156–158	27.5	15.6	9.88
5x	92.3	152–153.5	25.7	14.2	10.55
6x	81.5	152–153	22.9	14.8	5.27
7x	69.4	152.5–153.5	22.5	13.4	6.10
8x	61.1	153–153.5	19.5	13.8	4.67
9x	54.1	153–153.5	18.2	14.0	4.74
10x	45.5	153–154	18.5	13.6	4.90
11x	37.0	153–154	16.6	14.2	4.14
12x	32.5	153–153.5	16.0	13.7	7.39

[a]This weight does not include the residual amount of VCA not recovered by filtration but included in the next crystallization step. Melting points of VCA differ when it is recrystallized from alcohol-water (higher) or acetone-water (lower).

[b]Small amount of [^{14}C]VCA added as the internal standard.

[c]A sufficient amount of crystalline VCA was counted for 30–120 min in order to obtain ± 0.2 dpm/mg accuracy of the radioactivity determination; nd, not determined.

dary BA, its level ought to decrease dramatically in both cases.

Furthermore, the definitive proof for the hepatic synthesis of VCA was obtained from a small, but undoubtedly detectable, labeling after the intravenous administration of [1α,2α-$^3H_2$]cholesterol. It was also demonstrated by the efficient transformation of [24-^{14}C]CDC to [24-^{14}C]VCA.

As a result of the intravenous injections of tritiated cholesterol to opossum #11, about 9% of this radioactivity was secreted in the bile, but only 2.8% was present in the free carboxylic acid fraction. However, according to the reverse phase C_8 separation, most of this tritium was neither associated with VCA nor other BA and eluted as a broad peak directly after that of VCA. This was in contrast to the sharp elution of the "spike" of [24-^{14}C]CDCA added to the same separation. Nevertheless, after the initial drop in the specific radioactivity of VCA, a clearly detectable amount of tritium remained after twelve consecutive recrystallizations (Table 2). This low, but measurable incorporation was fully consistent with the expected dilution of the administered cholesterol and the known difficulty to label "newly biosynthesized cholesterol" representing the direct precursor of BA (19). With this available precursor, specially selected for its high specific activity, partial loss of the label evidently takes place; this is because 1α-hydroxylation ought to replace 1α-label and the intermediacy of 3-ketones in the biosynthesis can equilibrate part of the label at C-2.

In the case of the intravenous administration of [24-^{14}C]CDCA, at the early time of bile collection the secretion of this unconjugated precursor prevailed (Fig. 2). However, in the second hour the formation of free [24-^{14}C]VCA exceeded 50%. The incorporation of radioactivity to VCA was fully confirmed by its radiochromatographic purity as well as its specific radioactivity unchanged upon the multiple crystallization (Table 1, Fig. 4). Importantly, the original position of the label in the carboxyl group was retained in the newly biosynthesized VCA. It was demonstrated by the removal of carboxyl group to give C_{23} 23-bromo-1α,3α,7α-triacetoxy-5β-norcholane completely devoid of ^{14}C radioactivity. Such verification was necessary in order to exclude the involvement of side chain degradation and reincorporation of this label. As minor components, bile of *T. vulpecula* was found to contain several unique, short side-chain C_{23} and C_{22} BA[5], and such a possibility, although unlikely because of the high incorporation of radioactivity to VCA, could not be entirely excluded.

Taurine-conjugated VCA incorporated a small amount of radioactivity, but its estimated specific activity was not significantly different from that of the free acid (11 and 33 dpm/μg respectively). This may indicate that the conjugation, apparently of minor significance for this animal, lags behind the biosynthesis of VCA. On the other hand, although CDCA is the principal component of the taurine-conjugated fraction and only a minor component amongst free BA, merely a trace of the radioactivity was detected in tauro-CDCA of very low specific activity.

The efficient transformation of CDCA to VCA points to a plausible biosynthetic pathway leading to VCA (Fig. 1). The rapid decrease in the specific activity of CDCA, accurately measured for animal #42 (Table 1), indicates that only the pool of the free CDCA may really represent a quickly renewed precursor of VCA. Based on these observations, however, the introduction of 1α-hydroxyl group at an earlier biosynthetic stage cannot be entirely excluded. The late and very efficient hydroxylation of CDCA, which is clearly a primary BA, makes the primary classification of VCA comparable to the case of 3,6,7-hydroxylated BA. The late introduction of 1α and 6α hydroxyl groups, as the major hepatic pathway forming biliary BA, is evidently opposed to the "usual" 7α and 12α hydroxyl groups introduced in the earliest stages of BA biosynthesis. Fully consistent with the primary (or purely hepatic) origin of VCA is our recent observation that lithocholic acid (LCA) and its isomers are the major fecal BA of this marsupial (J. St. Pyrek and S. P. Lee, unpublished observations). Notably, this indicates the operation of a novel 1α,7α-bisdehydroxylation. Moreover, 1α-hydroxy-LCA which is the expected "secondary" product, assuming the definition of secondary BA as these resulting from 7-dehydroxylation, seems to be absent amongst fecal BA. It is present in the bile, however, as its 3-O-glucuronide together with the 3-O-glucuronide of LCA, raising obvious questions as to whether the hepatic rehydroxylation of these two acids at C-1 and C-7 takes place. Tentatively, we can presume that the major sequence involves the unconjugated CDCA, VCA (hepatic), LCA (microbial) which, after partial 1α-hydroxylation, are secreted in the bile as 3-O-glucuronides.

In the initial period of the bile collection, 7-dehydro-VCA, identified as a minor unconjugated BA, did not incorporate an appreciable amount of the radioactivity (Table 1). In the case of animals #21 and 22, the specific activity of this 7-keto-BA was low. However, in the case of animal #10, for which the bile was collected for a longer period, the difference in the specific activity of BA collected in the whole period was smaller, suggesting a slow hepatic transformation (probably a simple equilibration of both BA in a red-ox reaction) of VCA to 7-dehydro-VCA. Interestingly, another minor keto-BA, identified as 1-dehydro-VCA,[5] was apparently nonradioactive. Thus, the possible secondary origin of these two keto-BA cannot be excluded at this time.

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