

# Bioconversion of 3 $\beta$ -hydroxy-5-cholenoic acid into chenodeoxycholic acid by rat brain enzyme systems

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**Abstract** We have previously demonstrated that the rat brain contains three unconjugated bile acids, and chenodeoxycholic acid (CDCA) is the most abundantly present in a tight protein binding form. The ratio of CDCA to the other acids in rat brain tissue was significantly higher than the ratio in the peripheral blood, indicating a contribution from either a specific uptake mechanism or a biosynthetic pathway for CDCA in rat brain. In this study, we have demonstrated the existence of an enzymatic activity that converts 3 $\beta$ -hydroxy-5-cholenoic acid into CDCA in rat brain tissue. To distinguish marked compounds from endogenous related compounds, <sup>18</sup>O-labeled 3 $\beta$ -hydroxy-5-cholenoic acid, 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid, and 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid were synthesized as substrates for *in vitro* incubation studies. The results clearly suggest that 3 $\beta$ -hydroxy-5-cholenoic acid was converted to 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid by microsomal enzymes. The 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid was produced from 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid by the action of microsomal enzymes, and  $\Delta^4$ -3-oxo acid was converted to CDCA by cytosolic enzymes. These findings indicate the presence of an enzymatic activity that converts 3 $\beta$ -hydroxy-5-cholenoic acid into CDCA in rat brain tissue. Furthermore, this synthetic pathway for CDCA may relate to the function of 24S-hydroxycholesterol, which plays an important role in cholesterol homeostasis in the body.—Mano, N., Y. Sato, M. Nagata, T. Goto, and J. Goto. Bioconversion of 3 $\beta$ -hydroxy-5-cholenoic acid into chenodeoxycholic acid by rat brain enzyme systems. *J. Lipid Res.* 2004. 45: 1741–1748.

**Supplementary key words** biosynthesis • brain bile acid • cholesterol homeostasis • stable isotope dilution

Bile acids are synthesized in the liver from cholesterol by the action of hepatic enzymes and are excreted into the small intestine via the bile duct. In the intestinal lumen, the bile acids assist lipolysis and the absorption of fats by forming mixed micelles and then return to the liver upon absorption in the ileum and proximal colon. Because of their efficient hepatic uptake, bile acids are

present at low concentrations in the peripheral blood. Recent observations also indicate that the nuclear bile acid receptor, the farnesoid X receptor (FXR), regulates the bile acid pool by repressing the transcription of genes encoding hepatocyte transporters (1) and cholesterol 7 $\alpha$ -hydroxylase (2, 3), which is the rate-limiting enzyme for bile acid biosynthesis in the liver. The first step in the major hepatic bile acid biosynthetic pathway is the 7 $\alpha$ -hydroxylation of cholesterol. This product may or may not be 12 $\alpha$ -hydroxylated, and then conversion to a 5 $\beta$ -steroid nucleus and oxidation of the side chain are carried out to produce C<sub>27</sub> bile acid precursors.  $\beta$ -Oxidation of the side chain converts the precursors to the primary bile acids, cholic acid, and chenodeoxycholic acid (CDCA). It has also been reported that an alternative biosynthetic pathway is active in hepatocytes (4). It has been shown that 3 $\beta$ -hydroxy-5-cholenoic acid, which may be produced from 24,25-dihydroxycholesterol or 26-hydroxycholesterol, can undergo 7 $\alpha$ -hydroxylation and conversion to a 5 $\beta$ -steroid nucleus to produce CDCA in hamster (5), rabbit, and human livers (4).

Recently, we have demonstrated that three unconjugated bile acids, CDCA, cholic acid, and deoxycholic acid, exist in rat brain soluble fraction (6). In addition, CDCA, the most abundant bile acid in rat brain, tightly and non-covalently binds to some proteins in rat brain tissue. 24-Hydroxylated cholesterol, which is a possible precursor of 3 $\beta$ -hydroxy-5-cholenoic acid, has been detected at very high levels in the brain (7). In addition, Akwa et al. (8) have demonstrated that conversion of two neurosteroids, dehydroepiandrosterone and pregnenolone, into the corresponding 7 $\alpha$ -hydroxylated derivatives occurs in rat brain microsomal fractions. In addition, in an older publication, <sup>3</sup>H-labeled 3 $\beta$ -hydroxy-5-cholenoic acid was metabolized into lithocholic acid when incubated with normal guinea pig brain preparations (9).

Abbreviations: CDCA, chenodeoxycholic acid; ESI, electrospray ionization; FXR, farnesoid X receptor; HSD, hydroxysteroid dehydrogenase; IS, internal standard; LC/MS, liquid chromatography/mass spectrometry; R<sub>k</sub>, relative retention factor.

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In this study, we investigated whether or not an enzymatic activity that converts 3 $\beta$ -hydroxy-5-cholenoic acid into CDCA exists in rat brain tissue. Stable isotope-labeled substrates were synthesized and used as tracers to distinguish them from endogenous contaminants, and the incubation products were analyzed by liquid chromatography/mass spectrometry (LC/MS) coupled with electrospray ionization (LC/ESI-MS).

## EXPERIMENTAL PROCEDURES

### Materials and methods

CDCA and lithium were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and 3 $\beta$ -hydroxy-5-cholenoic acid, silver acetate, and BSA were purchased from Sigma Chemical Co. (St. Louis, MO).  $^{18}\text{O}$ -labeled water ( $\text{H}_2^{18}\text{O}$ , 95.4 atom%) was purchased from Euriso-Top (Saint-Aubin, France). 1,3-Dibromo-5,5-dimethylhydantoin was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 3,7-[ $^{18}\text{O}$ , $^2\text{H}$ ] $_2$ -CDCA and 12-oxolithocholic acid used as internal standards (ISs) were synthesized in our laboratory using previously reported methods (10). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). The potassium salts of bile acids and their derivatives were prepared by passing these substances through carboxymethyl Sephadex LH-20 ( $\text{K}^+$  form, 0.89 milliequivalents/g) lipophilic ion-exchange gel. A Sep-Pak  $\text{C}_{18}$  cartridge was purchased from Millipore (Milford, MA) and was washed successively with ethanol (20 ml), water (10 ml), 5% BSA aqueous solution (5 ml), and water (10 ml) before use. Water was purified with a Millipore water filtration system (Milli QUV Plus). All glassware was silanized with trimethylchlorosilane, and other chemicals and solvents were of analytical grade.  $^1\text{H}$  NMR analysis (300 MHz) was performed using a Hitachi FT-NMR R-3000 (Tokyo, Japan). Chemical shifts are given as  $\delta$  values with tetramethylsilane as an IS (*s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *dd*, double doublet; *m*, multiplet). Preparative HPLC was performed using an L-6200 pump (Hitachi) and 875-UV (Jasco, Tokyo, Japan) equipped with  $\mu$ Bondaspher 15  $\mu$  C18 (15  $\mu\text{m}$ , 7.8 mm inner diameter  $\times$  300 mm; Waters, Milford), which delivered 50 mmol/l sodium phosphate buffer (pH 7.0)-acetonitrile (12:5, v/v) as a mobile phase.

### LC/MS analysis

Analysis by LC/MS was performed using a JMS-LCmate (JEOL, Tokyo, Japan) double-focusing magnetic mass spectrometer equipped with an ESI probe under the negative ion detection mode. The resolution of the mass spectrometer was set at 750, and the voltages for the electrospray, orifice, and ring lens were  $-2.5$  kV,  $-40$  V, and  $-110$  V, respectively. The temperatures of the orifice and desolvating plate were  $150^\circ\text{C}$  and  $250^\circ\text{C}$ , respectively. Liquid chromatographic separation was performed using LC-10AD pumps (Shimadzu, Kyoto, Japan) and gradient elution on an L-Column (5  $\mu\text{m}$ , 2.1 mm inner diameter  $\times$  150 mm; Chemicals Inspection and Testing Institute, Tokyo, Japan) at a flow rate of 200  $\mu\text{l}/\text{min}$  with a 20 mmol/l ammonium acetate solution (pH 7.0 adjusted with ammonia)-acetonitrile mobile phase.

To compare the relative retention factors (*R*<sub>*k*</sub>) of enzymatic products with the *R*<sub>*k*</sub> of standards, we collected fractions corresponding to peaks on the chromatograms with the retention times of authentic bile acids. These fractions were mixed with cholic acid as an IS, and the *R*<sub>*k*</sub> relative to the IS ( $t_{\text{R}} - t_0/t_{\text{RIS}} - t_0$ ) were determined under four different separation conditions [20 mmol/l ammonium acetate solution (pH 4.0, 5.0, 6.0, and

7.0)-acetonitrile at 2:3, 2:3, 2:1, and 12:5 (v/v), respectively, for 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid; 1:1, 8:5, 2:1, and 12:5 (v/v), respectively, for 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid; and 9:11, 6:5, 9:5, and 11:5 (v/v), respectively, for CDCA].

### Synthesis of 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid

Because allyl alcohols are chemically unstable, the reaction and workup procedures for introducing a hydroxyl group into the C-7 position have to be performed under mild conditions. Lardy et al. (11, 12) have reported two methods for the introduction of a hydroxyl group at the C-7 position of 3 $\beta$ -hydroxy- $\Delta^5$  structures. The first method is the stereoselective reduction of a 7-carbonyl group (11), and the second method is the oxidative debromination of 7-bromo compounds, which are produced by allylic bromination, in the presence of acetic acid and silver acetate (12). Because the latter method is more general, allows easier handling, and yields a mixture of 7-bromides with a 7 $\alpha$ /7 $\beta$  ratio of  $\sim$ 1:1, we used the allylic bromination procedure with dibromantoin.

3 $\beta$ -Hydroxy-5-cholenoic acid (216 mg) was dissolved in tetrahydrofuran (2 ml), and diazomethane in diethyl ether was added at room temperature. After purification by column chromatography (*n*-hexane/ethyl acetate), the methyl ester of the starting cholenoic acid (225 mg) was dissolved in acetic anhydride (3 ml), pyridine (1 ml) was added, and the reaction was stirred at  $60^\circ\text{C}$  for 3 h. After adding ice-cold water to terminate the reaction, ethyl acetate was added to extract the acetate-methyl ester derivative. After successive washing with saturated sodium bicarbonate solution, brine, 5% hydrochloric acid solution, and brine, the organic layer was evaporated in vacuo to obtain a white crystal (251 mg).

The acetate-methyl ester derivative (200 mg) and sodium bicarbonate (216 mg) were added to 16 ml of *n*-hexane and the mixture was refluxed under a nitrogen atmosphere for 1 h. After the addition of 1,3-dibromo-5,5-dimethylhydantoin (97 mg), the mixture was further refluxed for 30 min. A precipitate was filtered out, and the filtrate was dried by evaporation in vacuo. Silver acetate (420 mg) was dissolved in a mixed solution of dichloromethane (5.2 ml) and acetic acid (1.28 ml) and was stirred at room temperature for 20 min. This mixture was then added to the residue containing methyl 7-bromo-3 $\beta$ -acetoxy-5-cholen-24-oate, and the resulting mixture was stirred in the dark for 30 min. After successive washing with water, 5% sodium bicarbonate, and water, the organic layer was evaporated in vacuo. After purification by column chromatography (*n*-hexane/ethyl acetate), hydrolysis was performed in a mixture of methanol (3 ml) and 10% potassium hydroxide (1 ml) at  $60^\circ\text{C}$  for 1 h. Salts were removed by reversed-phase column chromatography using 1 g of octadecylsilylated silica gel.

The purified product was methylated using diazomethane for 1 h. Next, silica gel column chromatography was carried out to separate the 7 $\alpha$ - and 7 $\beta$ -isomers of methyl 3 $\beta$ ,7-diacetoxy-5-cholen-24-oate. Recrystallization was carried out using acetone/*n*-hexane, and a white crystal (39 mg) was obtained. The stereoconfiguration at the 7-position was determined by  $^1\text{H}$  NMR analysis. The coupling constant of the 7 $\alpha$ -proton to the 8-proton must be 7–10 Hz in a doublet pattern because the angle between these protons is  $\sim$ 60 degrees. The signal of the 7 $\beta$ -proton must be a multiplet. The isomer that eluted first from the silica gel column chromatography had a doublet signal at 3.71 ppm with a coupling constant of 7.3 Hz, and the isomer that eluted second had a multiplet signal at 3.75 ppm. Therefore, the isomer that eluted second was identified as the 7 $\alpha$ -hydroxylated compound. This methyl 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholen-24-oate (35 mg) product was hydrolyzed in a mixed solution of 10% potassium hydroxide (1 ml) and methanol (0.5 ml) at  $60^\circ\text{C}$  for 1 h. After evaporation

of the methanol and neutralization with 10% acetic acid, desalting was carried out by reversed-phase chromatography. A white crystal (15 mg, 8.1% yield) was obtained and had the following NMR characteristics:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$ : 0.72 (3H, s, 18-H), 0.96 (3H, d, 21-H,  $J = 6.3$  Hz), 1.00 (3H, s, 19-H), 3.47 (1H, m, 3 $\alpha$ -H), 3.75 (1H, m, 7 $\beta$ -H), 5.53 (1H, dd, 6-H). The monoisotopic mass value for  $\text{C}_{24}\text{H}_{38}\text{O}_4$  [ $\text{M} - \text{H}$ ] $^-$  was 389.2696. The experimentally determined value was 389.2711.

#### Enzymatic preparation of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid

Preparation of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid was performed by enzymatic reaction using cholesterol oxidases, because this compound is very unstable both in acidic and alkaline solutions. Potassium 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid (18 mg), which was prepared by passage through carboxymethyl Sephadex LH-20 ion-exchange gel, was dissolved in 1.5 ml of 50 mmol/l sodium phosphate buffer (pH 7.4). Cholesterol oxidase (5 mg, 112 units) in the same buffer was then added, and the solution was incubated overnight at 37°C under a nitrogen atmosphere. The reaction mixture was subjected to desalting by reversed-phase column chromatography and was further purified by preparative HPLC on a  $\mu$ Bondaspher 15 $\mu$  C18-100Å column (7.8 mm inner diameter  $\times$  300 mm) using 50 mmol/l sodium phosphate buffer (pH 7.0)-acetonitrile (12:5, v/v) as the mobile phase. After desalting by reversed-phase column chromatography, the solvent was evaporated in vacuo to obtain a white powder (10 mg, 55.8% yield) with the following NMR characteristics:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$ : 0.76 (3H, s, 18-H), 0.96 (3H, d,  $J = 6.5$  Hz, 21-H), 1.23 (3H, s, 19-H), 3.92 (1H, m, 7 $\beta$ -H), 5.73 (1H, m, 4-H).

#### Preparation of 24- $^{18}\text{O}$ -labeled substrates

A tracer that can clearly distinguish target compounds from endogenous contaminants requires almost the same physico-chemical properties as the corresponding endogenous molecule; among the possible stable isotopes, the  $^{18}\text{O}$  atom is suitable for this purpose. The steroids used in this study have both of the hydroxyl groups on the steroid nucleus and have a carboxyl group at the side chain. However, a hydroxyl group at the C-3 position is not suitable for labeling, because of the possibilities of elimination and isotope effects during enzymatic reactions. Therefore, we introduced an  $^{18}\text{O}$  atom into the carboxyl group at the C-24 position according to the method previously reported by Strife and Murphy (13).

Methyl 3 $\beta$ -hydroxy-5-cholen-24-oate (18 mg) was dissolved in 2-propanol (0.5 ml), and 100  $\mu\text{l}$  of  $\text{H}_2^{18}\text{O}$  (95.4 atom%) and lithium (2 mg) were added. Hydrolysis was performed at 70°C for 1 h, and the reaction was terminated by the addition of 3 ml of 5% hydrogen chloride and extracted with ethyl acetate. The organic layer was evaporated in vacuo to obtain a white powder (15 mg, 80.7% yield) of 24- $^{18}\text{O}$ ]3 $\beta$ -hydroxy-5-cholenoic acid. 24- $^{18}\text{O}$ ]3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid was prepared in the same manner. 24- $^{18}\text{O}$ ]7 $\alpha$ -Hydroxy-3-oxo-4-cholenoic acid was prepared from 24- $^{18}\text{O}$ ]3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid using cholesterol oxidase. The ratios of the unlabeled deprotonated molecule to the labeled molecule were found to be 0.0752, 0.0554, and 0.0705 for 24- $^{18}\text{O}$ ]3 $\beta$ -hydroxy-5-cholenoic acid, 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid, and 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid, respectively.

#### Preparation of enzyme fractions

Male Wistar rats (150–200 g), fed a commercial pellet diet and water ad libitum, were used. Venous blood was collected and, after decapitation, whole brains (~1.8–2.0 g wet weight) were washed with saline and homogenized in 2 volumes of an ice-cold mixture of 50 mmol/l sodium phosphate buffer (pH 7.0) and glycerol (4:1, v/v). The homogenate was centrifuged at 600  $g$  for 10 min, followed by another centrifugation at 4,900  $g$  for 10 min.

The supernatant was further centrifuged at 24,000  $g$  for 10 min and then at 105,000  $g$  for 60 min. The final precipitate, corresponding to the microsomal fraction, was homogenized again with 4.5 ml of 50 mmol/l sodium phosphate buffer (pH 7.0). The supernatant fluids corresponding to the cytosolic fraction and the microsomal fraction were stored below 4°C. All procedures were carried out at 0–4°C.

#### 7 $\alpha$ -Hydroxylation of 3 $\beta$ -hydroxy-5-cholenoic acid

A 1:1 mixture of unlabeled and  $^{18}\text{O}$ -labeled 3 $\beta$ -hydroxy-5-cholenoic acid potassium salts (each 500 ng) was added to 100  $\mu\text{l}$  of 50 mmol/l sodium phosphate buffer (pH 7.0) containing 2.0 mmol/l EDTA and 2.0 mmol/l NADPH and preincubated at 37°C for 10 min. After addition of the rat brain microsomal preparation (200  $\mu\text{g}$  of protein per 100  $\mu\text{l}$  of the same buffer solution), the mixture was further incubated at 37°C for 15, 30, 60, and 120 min. The reaction was terminated by cooling the mixture on ice, and 12-oxolithocholic acid (253 ng/100  $\mu\text{l}$ ) as an IS and 5 ml of ethanol were added to the mixture. After centrifugation at 3,000 rpm for 10 min, the supernatant was evaporated in vacuo, 5 ml of 50 mmol/l sodium phosphate buffer (pH 7.0) was added, and the reaction products were subjected to solid-phase extraction using a Sep-Pak  $\text{C}_{18}$  cartridge. After washing with water (5 ml), the samples were eluted with 90% ethanol (5 ml). After evaporation of the solvent in vacuo, 10  $\mu\text{l}$  aliquots of the sample solution [100  $\mu\text{l}$  of 20 mmol/l ammonium acetate-ethanol (1:1, v/v)] were injected for LC/ESI-MS analysis.

#### Production of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid

A 1:1 mixture of unlabeled and  $^{18}\text{O}$ -labeled 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid potassium salts (each 500 ng) was added to 100  $\mu\text{l}$  of 50 mmol/l sodium phosphate buffer (pH 7.0) containing 2.0 mmol/l EDTA and 2.0 mmol/l  $\text{NAD}^+$  and preincubated at 37°C for 10 min. After addition of the rat brain microsomal fraction (200  $\mu\text{g}$  of protein per 100  $\mu\text{l}$  of the same buffer solution), the mixture was further incubated at 37°C for 7.5, 15, 30, and 60 min. The reaction was terminated by cooling the mixture on ice, and 12-oxolithocholic acid (253 ng/100  $\mu\text{l}$ ) and 5 ml of ethanol were added to the mixture. After centrifugation at 3,000 rpm for 10 min, the supernatant was evaporated in vacuo, 5 ml of 50 mmol/l sodium phosphate buffer (pH 7.0) was added, and the mixture was subjected to solid-phase extraction using a Sep-Pak  $\text{C}_{18}$  cartridge. After washing with water (5 ml), the samples were eluted with 90% ethanol (5 ml). After evaporation of the solvent in vacuo, 10  $\mu\text{l}$  aliquots of the sample solution [100  $\mu\text{l}$  of 20 mmol/l ammonium acetate-ethanol (1:1, v/v)] were injected for LC/ESI-MS analysis.

#### Conversion from 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid to CDCA

A DMSO solution (8  $\mu\text{l}$ ) of a 1:1 mixture of unlabeled and  $^{18}\text{O}$ -labeled 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid (each 2  $\mu\text{g}$ ) was added to 200  $\mu\text{l}$  of 50 mmol/l sodium phosphate buffer (pH 7.0) containing 10 mmol/l EDTA and 15 mmol/l NADPH. This solution was preincubated at 37°C for 10 min. After addition of the rat brain cytosolic fraction (4.8 mg of protein per 800  $\mu\text{l}$  of the same buffer solution), the mixture was further incubated at 37°C for 2, 4, 6, and 8 h. The reaction was terminated by cooling the mixture on ice, 3,7- $^{18}\text{O}$ , $^2\text{H}$ ] $_2$ -CDCA (4 ng/100  $\mu\text{l}$ ) was added, and the mixture was added dropwise into 500  $\mu\text{l}$  of 1.65 M Tris-HCl buffer (pH 8.6) containing 7.3 M guanidine and 0.03 M EDTA. After incubation at 37°C for 60 min, the reaction mixture was added to 5 ml of ethanol. After centrifugation at 3,000 rpm for 10 min, the supernatant was evaporated in vacuo, dissolved in 5 ml of 50 mmol/l sodium phosphate buffer (pH 7.0), and subjected to solid-phase extraction using a Sep-Pak  $\text{C}_{18}$  cartridge. Af-

ter washing with water (10 ml), the samples were eluted with 90% ethanol (5 ml). After evaporation of the solvent in vacuo, 50  $\mu$ l aliquots of the sample solution (100  $\mu$ l of 100 mmol/l ammonium acetate) were injected for LC/ESI-MS analysis.

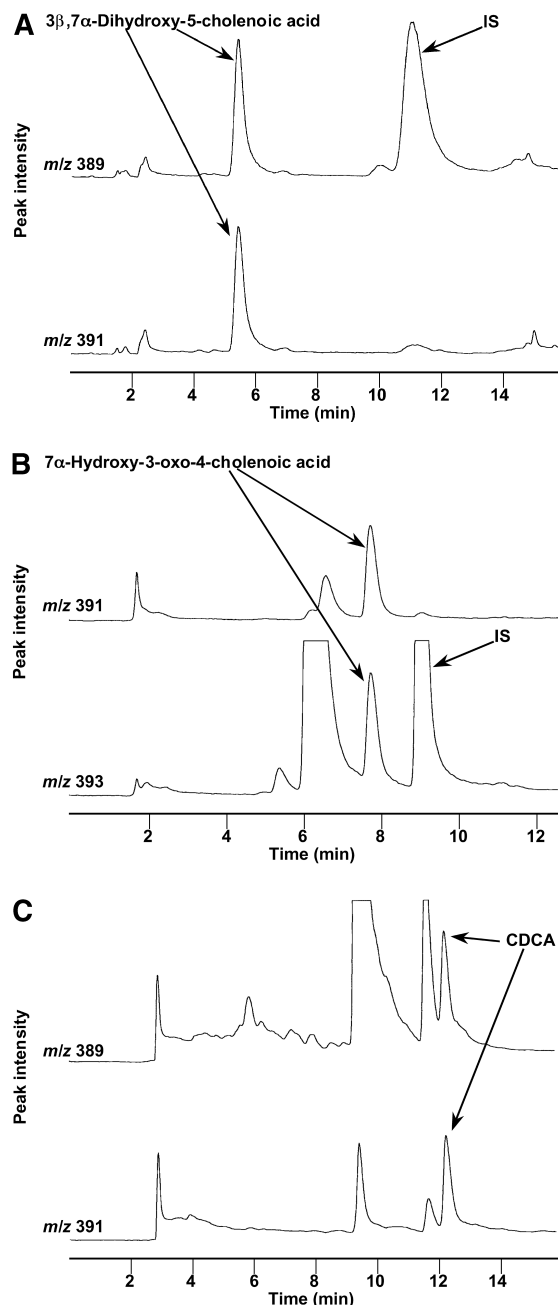
## RESULTS

### Enzymatic conversion of $3\beta$ -hydroxy-5-cholenoic acid to CDCA

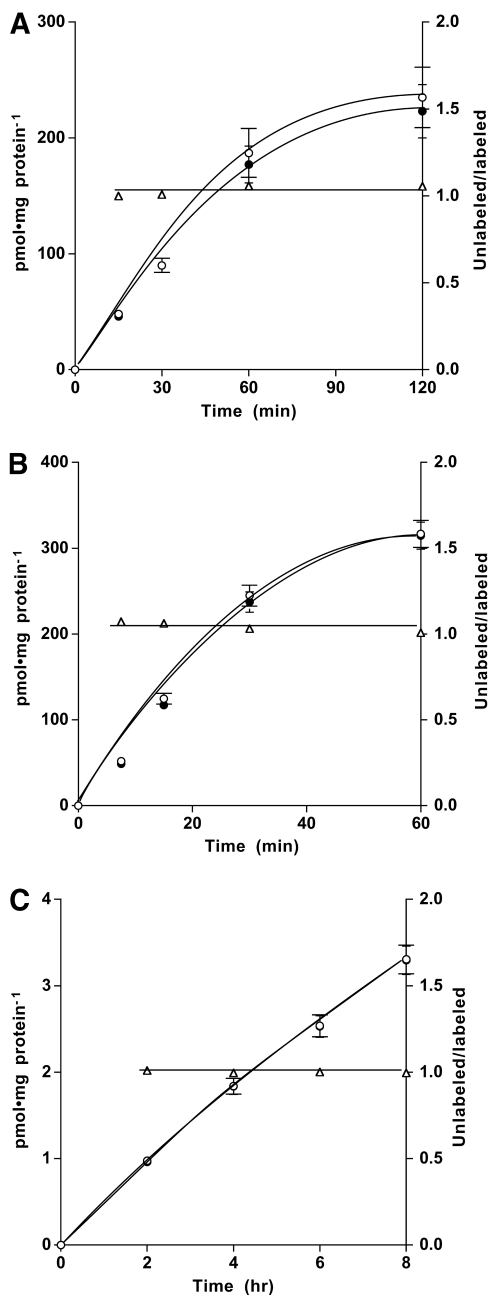
First, we incubated a 1:1 mixture of  $^{18}\text{O}$ -labeled and unlabeled  $3\beta$ -hydroxy-5-cholenoic acid with rat brain microsomal preparations in the presence of NADPH at  $37^\circ\text{C}$ . Two new peaks were produced in the incubation mixture at  $m/z$  389 and  $m/z$  391, and their retention times coincided with the authentic specimens of  $3\beta,7\alpha$ -dihydroxy-5-cholenoic acid (Fig. 1A). No such corresponding peaks were generated in the incubation mixture when we used a microsomal preparation inactivated by heating at  $80^\circ\text{C}$  for 20 min; this clearly suggested that the novel peaks were enzymatically produced. A time course profile of the  $7\alpha$ -hydroxylation of  $3\beta$ -hydroxy-5-cholenoic acid is shown in Fig. 2A. The enzymatic formation of the product increased until 120 min, and the ratio of  $^{18}\text{O}$ -labeled and unlabeled products was constant until the end of the reaction. This suggests that the products were produced from the substances added to the incubation mixture. In addition, both the substrate and product in the incubation mixture remained at 90% or greater purity for 2 h under the incubation condition.

Enzymatic conversion of  $3\beta,7\alpha$ -dihydroxy-5-cholenoic acid to  $7\alpha$ -hydroxy-3-oxo-4-cholenoic acid was performed using rat brain microsomal preparations in the presence of  $\text{NAD}^+$  at  $37^\circ\text{C}$ . Two peaks were clearly detected at  $m/z$  387 and  $m/z$  389, which correspond to  $7\alpha$ -hydroxy-3-oxo-4-cholenoic acid (Fig. 1B). The formation of the  $\Delta^4$ -3-keto steroid form increased in a time-dependent manner for 1 h, and the ratio of  $^{18}\text{O}$ -labeled to unlabeled product was almost constant during the incubation period (Fig. 2B). This fact suggests that the products were derived from the added substrates, a 1:1 mixture of  $^{18}\text{O}$ -labeled and unlabeled  $3\beta,7\alpha$ -dihydroxy-5-cholenoic acid. Although  $7\alpha$ -hydroxy-3-oxo-4-cholenoic acid is unstable under acidic or basic pH conditions, it was maintained at 90% or greater purity in the incubation mixture (pH 7.4) at  $37^\circ\text{C}$  for 1 h.

Enzymatic conversion of  $7\alpha$ -hydroxy-3-oxo-4-cholenoic acid to CDCA by a cytosolic preparation of rat brain was carried out in the presence of NADPH at  $37^\circ\text{C}$ . This reaction took place with lower activity than either  $7\alpha$ -hydroxylation or  $\Delta^4$ -3-keto formation. Peaks corresponding to CDCA were observed in the selected ion monitoring chromatograms at  $m/z$  391 and  $m/z$  393 (Fig. 1C). Time-dependent formation of CDCA was observed, and the amount of product increased until 8 h, with an almost constant ratio of unlabeled to  $^{18}\text{O}$ -labeled products (Fig. 2C). Taken together, these results suggest that  $3\beta$ -hydroxy-5-cholenoic acid can be converted to CDCA in rat brain tissue through  $3\beta,7\alpha$ -dihydroxy-5-cholenoic acid and  $7\alpha$ -hydroxy-3-oxo-4-cholenoic acid intermediates.



**Fig. 1.** Typical selected ion monitoring chromatograms of enzymatic products from  $3\beta$ -hydroxy-5-cholenoic acid (A),  $3\beta,7\alpha$ -dihydroxy-5-cholenoic acid (B), and  $7\alpha$ -hydroxy-3-oxo-4-cholenoic acid (C). Chromatographic conditions were as follows: column, L-column ( $5\ \mu\text{m}$ ,  $2.1\ \text{mm}$  inner diameter  $\times$   $150\ \text{mm}$ ); mobile phase, (A) step gradient, 20 mmol/l ammonium acetate solution (adjusted to pH 7.0 with ammonia)-acetonitrile (12:5, v/v) for the initial 10 min and (4:5, v/v) for the following 10–30 min, (B) linear gradient, 20 mmol/l ammonium acetate solution (adjusted to pH 7.0 with ammonia)-acetonitrile (7:3, v/v) to (1:1, v/v) over 13 min, and (C) 20 mmol/l ammonium acetate solution (adjusted to pH 7.0 with ammonia)-acetonitrile (7:3, v/v) to (1:1, v/v) over 13 min and (1:1, v/v) to (1:2, v/v) over 3 min; flow rate, 0.2 ml/min; electrospray voltage,  $-2.5\ \text{kV}$ ; orifice voltage,  $-40\ \text{V}$ ; ring lens voltage,  $-110\ \text{V}$ . CDCA, chenodeoxycholic acid; IS, internal standard.



**Fig. 2.** Time course of the production of  $3\beta,7\alpha$ -dihydroxy-5-cholenic acid (A),  $7\alpha$ -hydroxy-3-oxo-4-cholenic acid (B), and CDCA (C). Left y axis, amount of product in the incubation mixture; right y axis, ratio of unlabeled to  $^{18}\text{O}$ -labeled product; open circles, product from unlabeled substrate; closed circles, product from  $^{18}\text{O}$ -labeled substrate; open triangles, ratio of unlabeled to  $^{18}\text{O}$ -labeled product.  $n=3$ , the results of the amount of product, was expressed as mean  $\pm$  SD.

Bile acids and related compounds have a characteristic chromatographic behavior attributable to the number and position of the hydroxyl group on the steroid nucleus; this is very useful for the identification of trace amounts of these compounds in biological fluids (14). Therefore, the fractions corresponding to the enzymatic products were separately collected and subjected to LC/ESI-MS analysis using four mobile phases at different pH

**TABLE 1.** Retention factors of the authentic specimens and the incubation products relative to cholic acid:  $3\beta,7\alpha$ -dihydroxy-5-cholenic acid

	Retention Factors Relative to Cholic Acid			
	pH 4 <sup>a</sup>	pH 5 <sup>b</sup>	pH 6 <sup>c</sup>	pH 7 <sup>d</sup>
Standard	1.24	1.33	1.13	0.89
Incubation product	1.24	1.33	1.14	0.89

<sup>a</sup> 20 mmol/l ammonium acetate buffer (pH 4.0)-acetonitrile (2:3, v/v).

<sup>b</sup> 20 mmol/l ammonium acetate buffer (pH 5.0)-acetonitrile (2:3, v/v).

<sup>c</sup> 20 mmol/l ammonium acetate buffer (pH 6.0)-acetonitrile (2:1, v/v).

<sup>d</sup> 20 mmol/l ammonium acetate buffer (pH 7.0)-acetonitrile (12:5, v/v).

values. The  $R_k$  values of the  $3\beta,7\alpha$ -dihydroxy-5-cholenic acid,  $7\alpha$ -hydroxy-3-oxo-4-cholenic acid, and CDCA relative to the  $R_k$  value of the IS, cholic acid, were identical (Tables 1–3).

## DISCUSSION

This study demonstrates that  $3\beta$ -hydroxy-5-cholenic acid can undergo enzymatic conversion to CDCA in rat brain tissue (Fig. 3), and the apparent velocity of  $7\alpha$ -hydroxylation by a rat brain microsomal preparation was 6 pmol/mg protein/min. In the same incubation condition using rat liver microsomal preparation as an enzyme source, the activity of  $7\alpha$ -hydroxylation was 20 times higher than using rat brain tissue (data not shown). Pregnenolone and dehydroepiandrosterone, two major neurosteroids, are converted in an NADPH-dependent manner into  $7\alpha$ -hydroxylated derivatives, but not  $7\beta$ -hydroxylated derivatives, by the action of hepatic oxysterol  $7\alpha$ -hydroxylase (cyp7b) in rat and mouse brain (8, 15). Furthermore, recombinant cyp7b more efficiently catalyzed the formation of steroids possessing  $3\beta$ -hydroxy- $\Delta^5$  structures than other steroids (15). The activity of cyp7b decreased by 33% in the presence of cholesterol  $7\alpha$ -hydroxylase (cyp7a) and was inhibited by cholesterol,  $7\alpha$ - and  $7\beta$ -

**TABLE 2.** Retention factors of the authentic specimens and the incubation products relative to cholic acid:  $7\alpha$ -hydroxy-3-oxo-4-cholenic acid

	Retention Factors Relative to Cholic Acid			
	pH 4 <sup>a</sup>	pH 5 <sup>b</sup>	pH 6 <sup>c</sup>	pH 7 <sup>d</sup>
Standard	1.72	1.91	1.54	1.36
Incubation product	1.71	1.91	1.55	1.36

<sup>a</sup> 20 mmol/l ammonium acetate buffer (pH 4.0)-acetonitrile (1:1, v/v).

<sup>b</sup> 20 mmol/l ammonium acetate buffer (pH 5.0)-acetonitrile (8:5, v/v).

<sup>c</sup> 20 mmol/l ammonium acetate buffer (pH 6.0)-acetonitrile (2:1, v/v).

<sup>d</sup> 20 mmol/l ammonium acetate buffer (pH 7.0)-acetonitrile (12:5, v/v).

TABLE 3. Retention factors of the authentic specimens and the incubation products relative to cholic acid: chenodeoxycholic acid

	Retention Factors Relative to Cholic Acid			
	pH 4 <sup>a</sup>	pH 5 <sup>b</sup>	pH 6 <sup>c</sup>	pH 7 <sup>d</sup>
Standard	3.43	3.90	4.34	3.70
Incubation product	3.43	3.89	4.33	3.70

<sup>a</sup> 20 mmol/l ammonium acetate buffer (pH 4.0)-acetonitrile (9:11, v/v).

<sup>b</sup> 20 mmol/l ammonium acetate buffer (pH 5.0)-acetonitrile (6:5, v/v).

<sup>c</sup> 20 mmol/l ammonium acetate buffer (pH 6.0)-acetonitrile (9:5, v/v).

<sup>d</sup> 20 mmol/l ammonium acetate buffer (pH 7.0)-acetonitrile (11:5, v/v).

hydroxycholesterol (16). These facts demonstrate that the pathway that includes *cyp7b* metabolism was not the major route of bile acid biosynthesis in the liver. Murine and human cDNAs and genes that encode a second oxysterol 7 $\alpha$ -hydroxylase have also been cloned and characterized (17). CYP39A1, expressed in the liver, has a preference for 24-hydroxycholesterol, and its hepatic expression levels have no relation with dietary cholesterol and bile acids. The activity of 7 $\alpha$ -hydroxylation in male mice is  $\sim$ 20 pmol/mg protein/min. *Cyp7a*, the rate-limiting enzyme in hepatic bile acid biosynthesis, was isolated from a rat liver microsomal preparation (18), and the *cyp7a* mRNA was expressed only in liver tissue (19). In contrast, *cyp7b* mRNA expression was estimated in multiple human tissues, and the order of abundance of mRNA levels after normalization to a  $\beta$ -actin signal was liver > brain = kidney = pancreas > heart > lung > skeletal muscle > placenta (20). Therefore, *cyp7b* expressed in rat brain may catalyze the 7 $\alpha$ -hydroxylation of 3 $\beta$ -hydroxy-5-cholenoic acid, which has been clearly demonstrated in this study. However, in the presence of equimolar pregnenolone or dehydroepiandrosterone, the 7 $\alpha$ -hydroxylation of 3 $\beta$ -hydroxy-5-cholenoic acid was not inhibited during the preliminary experiments (data not shown).

The rat brain microsomal preparation also has an activity that converts 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid to 7 $\alpha$ -

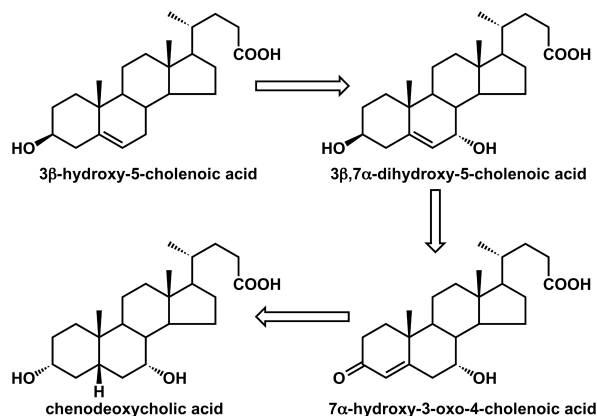



Fig. 3. Possible mechanism for the synthesis of CDCA from 3 $\beta$ -hydroxy-5-cholenoic acid by rat brain tissue.

hydroxy-3-oxo-4-cholenoic acid at a rate of 19 pmol/mg protein/min. 3 $\beta$ -Hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase (3 $\beta$ -HSD), which plays a role in the synthesis of neurosteroids, was expressed at the mRNA and protein levels in rat brain, at a particularly high level in the cerebellum, and an enzymatic activity for conversion of 3 $\beta$ -hydroxy- $\Delta^5$  derivatives into the corresponding  $\Delta^4$ -3-keto steroids was demonstrated (21). In hepatocytes, bile acids bind to cytosolic bile acid binding protein, which is a member of the monomeric reductase gene family and has dihydrodiol dehydrogenase activity but does not have activity toward bile acids in human (22). In contrast, the major bile acid binder is identical to 3 $\alpha$ -HSD in rat hepatocytes (23). The bile acid binding protein may assist in the rapid intracellular transport of bile acids from the sinusoidal pole to the canalicular pole of the cell. Unconjugated CDCA found in rat brain tissue is tightly bound to a cytosolic protein (6), which has caused great difficulty in the detection of brain bile acids until now. Weil (24) noted that taurocholic acid caused marked demyelination in vitro and proposed that some type of bile acid might act as a natural demyelinating agent; furthermore, this agent might be formed biosynthetically within the brain or enter the brain from the bloodstream. Strangely, a high dose of <sup>14</sup>C-labeled cholic acid injected intracerebrally could not be detected in appreciable amounts in central nervous system tissue within 1 week of the injection (9). To speculate upon these previous findings, the brain bile acid binding protein may play a key role in the rejection of toxicity based on detergent effects, including demyelination, and may function as an intracellular transporter.

The rat brain cytosolic preparation contained an enzymatic activity for the conversion of 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid to CDCA; however, this activity, which was 14 fmol/mg protein/min, was much lower than the other two described metabolic activities. Rat liver  $\Delta^4$ -3-ketosteroid 5 $\beta$ -reductase was purified 230-fold from a 100,000 g supernatant; this enzyme catalyzed NADPH-dependent reactions and acted with a broad range on various steroids possessing  $\Delta^4$ -3-keto structures (25). Although the metabolism of progesterone and testosterone has been demonstrated to produce 5 $\alpha$ -saturated derivatives in the brain of the previable human fetus (26), to the best of our knowledge there has been no report of the production of 5 $\beta$ -saturated metabolites from  $\Delta^4$ -3-keto steroids. However, it has been reported that <sup>3</sup>H-labeled 3 $\beta$ -hydroxy-5-cholenoic acid can be metabolized to lithocholic acid via a  $\Delta^4$ -3-keto derivative when incubated with normal guinea pig brain preparations (9). Significant amounts (1.0–18.8 nmol/g wet weight) of bile acids were detected in brain biopsies of patients immediately after death from fulminant hepatic failure, whereas no bile acids were detected in brain biopsies from patients after death without evidence of liver disease (27). These observations suggest that brain bile acids, which may act as demyelinating agents or produce other toxic effects (24), are closely regulated.

Great amounts of 24S-hydroxycholesterol exist in rat brain microsomal fractions (7). This substance undergoes 25-hydroxylation in rat astrocytes to produce 24,25-dihy-

droxycholesterol (7), which may be readily converted to a C-24 acid by the 100,000 g supernatant of liver homogenates (28). These facts may indicate that 24-hydroxycholesterol acts as an intermediate of the synthetic pathway from cholesterol to 3 $\beta$ -hydroxy-5-cholenoic acid. 24S-Hydroxycholesterol synthesized in the brain is excreted into the peripheral bloodstream for transfer to the liver (29, 30) and is then converted into bile acids in human hepatocytes (31). In addition, increased levels of oxysterols, such as 24-hydroxycholesterol, induce liver bile acid biosynthesis and degradation of cholesterol via binding to the oxysterol receptor (32). Conversely, bile acids synthesized by activation of cyp7a bind to FXR and suppress cholesterol 7 $\alpha$ -hydroxylation by negative feedback to cyp7a; this process maintains cholesterol and bile acid homeostasis (33). Although the function of brain bile acids is unknown and the brain bile acid binding protein has not been identified, it is possible that brain bile acids play a role in bile acid and cholesterol homeostasis, including the function of 24S-hydroxycholesterol synthesized in brain.

In conclusion, we have demonstrated the enzymatic conversion of 3 $\beta$ -hydroxy-5-cholenoic acid to CDCA through 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid and 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid intermediates in rat brain tissue. The first metabolic step, 7 $\alpha$ -hydroxylation, was dependent on the presence of NADPH and the production of 7 $\alpha$ -hydroxylated neurosteroid by cyp7b. Furthermore, NAD<sup>+</sup>-dependent 3 $\beta$ -HSD isomerase activities that acted on 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholenoic acid were identified in a rat brain microsomal preparation. 7 $\alpha$ -Hydroxy-3-oxo-4-cholenoic acid was converted in vitro to CDCA by a rat brain cytosolic preparation, although this activity was considerably lower. In addition, the isotope dilution method using stable isotope-labeled substrates was able to distinguish enzymatic products from other biological contaminants. 

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