Transport of Fluorescent Bile Acids by the Isolated Perfused Rat Liver: Kinetics, Sequestration, and Mobilization

Fernando Holzinger, Claudio D. Schteingart, Huong-Thu Ton-Nu, Carolina Cerrè, Joseph H. Steinbach, Hong-Zen Yeh, and Alan F. Hofmann

Hepatocyte transport of six fluorescent bile acids containing nitrobenzoxadiazolyl (NBD) or a fluorescein derivative on the side chain was compared with that of natural bile acids using the single-pass perfused rat liver. Compounds were infused at 40 nmol/g liver · min for 15 minutes; hepatic uptake and biliary recovery were measured; fractional extraction, intrinsic basolateral clearance, and sequestration (nonrecovery after 45 minutes of additional perfusion) were calculated. Fluorescent bile acids were efficiently extracted during the first 3 minutes (70%-97%), but net extraction decreased with time mostly because of regurgitation into the perfusate. For cholylglycine and ursodeoxycholylglycine (UDC-glycine), extraction was 94% to 99%, and regurgitation did not occur. Intrinsic hepatic clearance of fluorescent bile acids (2-7 mL/g liver · min) was lower than that of cholylglycine (9.0 ± 0.6; mean ± SD) and UDC-glycine (21.4 ± 0.4). Sequestration at 60 minutes was 8% to 26% for fluorescent bile acids with a cholyl moiety (cholylglycylaminofluorescein [CGamF], cholyllysylfluorescein [C-L-F], cholyl-[Nε-NBD]-lysine [C-L-NBD], and cholylaminofluorescein [CamF]), 32% for ursodeoxycholylaminofluorescein (UDCamF), and 88% for ursodeoxycholyl-(Nε-NBD)-lysine (UDC-L-NBD). Cholylglycine and UDC-glycine had <3% retention. Biliary secretion of sequestered UDCamF, but not of UDC-L-NBD, was induced by adding dibutyryl cyclic adenosine monophosphate (DBcAMP) to the perfusate, possibly by translocation to the canaliculus of pericanalicular vesicles containing fluorescent bile acids. Biliary secretion of UDC-L-NBD, but not of UDCamF, was induced by adding cholyltaurine or UDC-taurine, possibly by inhibition of binding to intracellular constituents or of transport into organelles. It is concluded that fluorescent bile acids are efficiently transported across the basolateral membrane, but in contrast to natural conjugated bile acids, are sequestered in the hepatocyte (UDC derivatives > cholyl derivatives). Two modes of hepatic sequestration of fluorescent bile acids were identified. Fluorescent bile acids may be useful to characterize sequestration processes during bile acid transport through the hepatocyte. (HEPATOLOGY 1998; 28:510-520.)

Bile acids tagged with a fluorophore (fluorescent bile acids) have been used as surrogates for natural bile acids to visualize bile acid transport in hepatocytes,[1-12] cholangiocytes,[13] or nonhepatocytes transfected with mRNA for *ntcp*,[14] a bile salt transporter present in the basolateral membrane of the hepatocyte. Our laboratory has synthesized two classes of fluorescent bile acids with the fluorophore on the side chain. In the first, aminofluorescein (amF) is coupled to the carboxylic acid group of a glycine-conjugated or unconjugated bile acid. In the second, nitrobenzoxadiazolyl (NBD) is coupled to the ε-amino group of a lysine-conjugated bile acid.

We have recently characterized the hepatic and intestinal transport of several representatives of these two classes of fluorescent bile acids in the rat.[15] Transport of these molecules was compared with that of two natural conjugated bile acids (cholylglycine [glycocholate] and cholyltaurine [taurocholate]), as well as that of fluorescein. We found that these fluorescent bile acids, like their corresponding natural conjugated bile acids, were transported vectorially across the hepatocyte without undergoing appreciable biotransformation. Hepatocyte transport of fluorescent bile acids was less efficient than that of cholylglycine or cholyltaurine, and both the chemical nature of the fluorophore as well as that of the bile acid moiety influenced the magnitude of canalicular transport. We also found that several of the fluorescent bile acids differed from natural bile acids in being cholestatic rather than choleretic.

Whole-animal studies are useful for defining the overall toxicity of a molecule, and its route of elimination, i.e., whether it is hepatic or renal, or both. They also provide information on overall hepatic transport rate, and whether biotransformation occurs before elimination in bile or urine. However, such studies provide no information on first-pass

extraction or on the rate of canalicular secretion in relation to the amount of compound within the hepatocyte.

In the present study, we have characterized the transport of six such fluorescent bile acids using the isolated rat liver perfused in single-pass fashion. In addition to the five compounds previously characterized by us in the whole animal,[15] we have synthesized and examined the transport of cholylfluoresceinthiocarbamoyllysine, a molecule prepared by Mills et al. and named cholyllysylfluorescein (C-L-F) by this group.[6] According to these workers, this molecule is transported by the liver in a manner identical to that of cholyltaurine.[6,16] We have compared the kinetics of transport of these six fluorescent bile acid derivatives with those of three natural bile acids (cholylglycine, ursodeoxycholylglycine [UDC-glycine], and cholyltaurine), and with those of norursodeoxycholic acid (norUDCA), a molecule that undergoes cholehepatic shunting in the isolated perfused liver.[17,18] We have also examined the transport of fluorescein, a commonly used fluorescent organic anion.

Because we observed that certain fluorescent bile acids, in contrast to the natural bile acids, were sequestered within the liver, experiments were performed to test whether these sequestered fluorescent bile acids could be mobilized, as evidenced by biliary secretion, when dibutyryl cyclic adenosine monophosphate (DBcAMP) or a conjugated bile acid was added to the perfusate.

MATERIALS AND METHODS

Experimental Design. Two perfusion protocols were used. In the first, after a baseline period of 20 minutes, the fluorescent test substance, or a bile acid, was perfused at a constant rate for 15 minutes; perfusion was then continued with perfusate lacking the test compound for an additional 45 minutes. The concentration of infused compound was measured separately in the entering and draining perfusate and in bile. In contrast to natural conjugated bile acids, for which steady-state fractional hepatic extraction was observed after 1 minute of perfusion time, all fluorescent bile acids, norUDCA, and fluorescein showed a time-dependent decrease in hepatic fractional extraction. Accordingly, our experiments were performed under an "approach to steady-state condition" for these compounds.

In the second protocol, an agent (DBcAMP or a conjugated bile acid) was added to the perfusate in an attempt to induce biliary secretion (mobilization) of fluorescent bile acids that were sequestered within the liver (presumably in the hepatocytes). The DBcAMP was added in an attempt to induce pericanalicular vesicles to fuse with the canalicular membrane; the conjugated bile acid was added in an attempt to displace competitively sequestered fluorescent bile acids from putative intracellular bile acid binding sites and/or to inhibit transport of the fluorescent bile acids into a sequestering space.

Chemicals and Radiochemicals. Six fluorescent bile acids containing the fluorophore on the side chain were used in this study. Their chemical structures are shown in Fig. 1. Trivial names, molecular weights, and relative retention times by high-performance liquid chromatography for five of these compounds have been published previously.[15] Three of the fluorescent bile acids were tagged with amF. The bile acid moiety consisted of cholylglycine (CGamF), of cholic acid (CamF), or of ursodeoxycholic acid (UDCamF). In the remaining three, the bile acid moiety was cholyl- or ursodeoxycholyl-lysine, and the fluorophore was coupled to the ε-amino group of the lysine. In two of these, the fluorophore was NBD (C-L-NBD and UDC-L-NBD); in the third, it was fluoresceinthiocarbamoyl-. This last molecule has the correct chemical name of Nα-cholyl-Nε-(5-fluoresceinthiocarbamoyl)-lysine; however, in this article, it will be termed cholyllysylfluorescein (C-L-F), following the recommendation of Mills et al.[6] These three molecules can be considered to be NBD-amino-*n*-butyl or fluoresceinthiocarbamoyl-amino-*n*-butyl derivatives of the corresponding glycine conjugate.

Fluorescent bile acids were synthesized and purified by column chromatography as described previously.[15,19,20] C-L-F was synthesized by a method based on that reported by Mills et al.[6] Briefly, Nα-cholyllysine (117 mg) was dissolved in 10 mL of methanol containing 0.5 mL of 10% wt/vol KOH in water (4 eq). Fluorescein isothiocyanate (102 mg, 1.2 meq) was added, and the reaction mixture (protected from light) was stirred at room temperature for 24 hours. The reaction mixture was diluted with 100 mL of water, acidified with HCl to pH 1, and extracted twice with ethyl acetate. The combined organic layers were washed with 200 mL 15% NaCl, reduced to dryness using a rotary evaporator, and the residue dissolved in 3 mL of water containing 3 eq NaOH. This solution was applied to a C_{18} reversed-phase cartridge (Mega Bond Elut, Varian, Harbor City, CA). The cartridge was washed with water, and the compound eluted with a gradient of methanol in water. The compound was eluted at a concentration of 25% to 35% vol/vol methanol. The fractions containing pure C-L-F (assessed by thin-layer chromatography on silica gel layers using a solvent system of chloroform:methanol:acetic acid, 80:20:4 [vol:vol]) were pooled, and the solvent removed. The final compound (yield, 120 mg) was
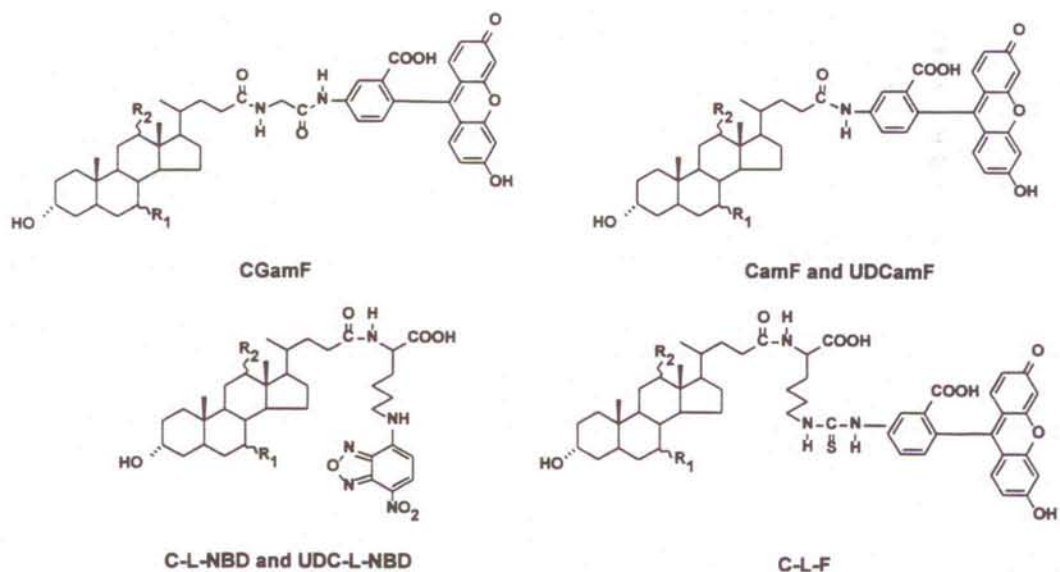

FIG. 1. Chemical structures of fluorescent bile acid derivatives. In the cholyl-derivatives, R_1 and R_2 denote α-hydroxy groups; in the ursodeoxycholyl-derivatives, R_1 is a β-hydroxy group, and no hydroxy group is present at R_2.

CGamF

CamF and UDCamF

C-L-NBD and UDC-L-NBD

C-L-F

98% pure by high-performance liquid chromatography using a system described for natural bile acids, with detection at 280 nm[21]; the relative retention time (versus cholyltaurine) was 1.91. Analysis by electrospray mass spectrometry using a Perkin Elmer SCIEX API-3 mass spectrometer at The Scripps Research Institute Mass Spectrometry Lab (La Jolla, CA) showed a pseudomolecular ion $[MH]^+$ at m/z 926 in the positive mode and a pseudomolecular ion $[M-H]^-$ at m/z 924 in the negative mode, in agreement with the calculated value 925.4 for the expected compound $C_{51}H_{63}N_3H_{11}S$. The structure of the compound was also confirmed by proton nuclear magnetic resonance (data not shown).

Cholyltaurine and cholylglycine were purchased from Sigma Chemicals (St. Louis, MO). UDC-glycine was synthesized by the method of Tserng et al.[22] and purified by column chromatography.[20] NorUDCA was prepared from UDCA.[23]

Fluorescein sodium salt (F) and $N^6,2'$-O-dibutyryladenosine 3':5'cyclic monophosphate (DBcAMP) were purchased from Sigma Chemicals. Compounds were stated by the manufacturer to have a purity exceeding 97% and were used without further purification.

[23-^{14}C]-norUDCA (10 mCi/mmol), [24-^{14}C]-UDC-glycine (15 mCi/mmol), and [22,23-^{3}H]-cholyltaurine (58 Ci/mmol) were synthesized as previously reported.[17,24] The final compounds, after purification by preparative thin-layer chromatography, had a radiochemical purity of 98% by zonal scanning. [1-^{14}C]-Cholylglycine (52.5 mCi/mmol) was purchased from New England Nuclear Du Pont (Boston, MA); the compound was stated to have a radiochemical purity of 99% by the manufacturer and was used without further purification.

Isolated Liver Perfusion. Animal studies were approved by the Committee on Animal Studies of the University of California, San Diego.

Isolated livers from male Sprague-Dawley rats (270-320 g) (Charles Rivers Laboratories, Wilmington, MA) were perfused *in situ*, in single-pass fashion, as described by Wolkoff et al.[25] After induction of anesthesia by intraperitoneal injection of pentobarbital (Nembutal, 7.5 mg/100 g body wt, Abbott Laboratories, North Chicago, IL), the abdomen was opened by a midline and subcostal incision, and a biliary fistula was constructed using PE-10 tubing (Clay Adams, Parsippany, NJ). After injection of 100 Units heparin (Elkins-Sinn, Inc., Cherry Hill, NJ) into the inferior vena cava, the portal vein was cannulated using a 16-gauge intravenous catheter (OptiVa, Critikon, Tampa, FL), and liver perfusion was begun immediately with oxygenated (95% O_2-5% CO_2) Krebs-Ringer bicarbonate buffer solution (pH 7.4). Liver isolation was completed by cannulation of the hepatic vein (14-gauge intravenous catheter [Cathlon IV, Critikon, Tampa, FL]) after thoracotomy. The perfusate flow was maintained at 36 ± 1 mL/min, and the portal vein pressure was kept constant at 12 to 13 cm H_2O. The perfusate temperature was 38°C. Liver temperature was maintained at 38 ± 0.5°C by the heated perfusate as well as by a temperature-controlled heating lamp with a thermistor probe (Yellow Springs Instruments, Yellow Springs, OH) inserted between the lobes of the liver. Viability of perfused livers was assessed by a homogenous appearance of the liver surface and an initial bile flow rate of at least 50 µL/5 min. Bile was collected every 5 minutes in preweighed plastic vials throughout the entire experiment.

The Krebs-Ringer bicarbonate solution consisted of: NaCl (119 mmol/L), NaHCO$_3$ (25 mmol/L), KCl (4.74 mmol/L), KH$_2$PO$_4$ (1.19 mmol/L), MgSO$_4 \cdot$ 7H$_2$O (1.19 mmol/L), and CaCl$_2 \cdot$ 2H$_2$O (2.6 mmol/L). In addition, D-glucose (5.55 mmol/L) and sodium nitroprusside (5.9 mg/L), a vasodilator, were added to the Krebs-Ringer bicarbonate solution.

In experiments using the first protocol, after a 20-minute stabilization period, a fluorescent bile acid, a radiolabeled bile acid (with carrier bile acid), or fluorescein were infused from a syringe pump (Harvard Apparatus Co. Inc., Millis, MA) into the perfusion line via a side-arm port 60 cm upstream from the portal vein cannula. Each compound was dissolved in 15 mL 150 mmol/L NaCl (pH adjusted to 8 with 0.1 N NaOH) and infused for 15 minutes.

The concentration in the entering perfusate (collected at the beginning and end of the perfusion by a PE-10 tube side-arm port just before the portal cannula) was 10 ± 1 µmol/L, resulting in an infusion rate of ≈40 nmol/g liver · min. The perfusate effluent was collected at 30-second intervals over the first 20 minutes in preweighed vials, followed by 30-second intervals every 5 minutes for the remaining 40 minutes until the end of the experiment. At the end of the experiment, the liver was removed surgically and its wet weight was determined.

In experiments using the second protocol, DBcAMP or conjugated bile acids were added to the perfusate. For experiments with DBcAMP, the liver was perfused with 100 µmol/L DBcAMP for 20 minutes (from the beginning of the perfusion). Subsequently, the DBcAMP was coinfused with selected fluorescent bile acids, each at a concentration of 10 ± 1 µmol/L. For experiments with conjugated bile acids, cholyltaurine or UDC-taurine were coinfused with the fluorescent bile acid, each at a concentration of 10 ± 1 µmol/L.

Analytical Methods. Concentrations of fluorescent bile acids and fluorescein in bile and perfusate were determined spectrophotometrically (Shimadzu UV-12016, Shimadzu Scientific Instruments Inc., Columbus, MD), as previously described,[15] because of the strong effects of pH and microenvironment on fluorescence emission.[10] The absorbance of fluorescein-containing compounds was determined at 492 nm. Extinction coefficients (in units of 10^4 mol/L^{-1} cm^{-1}) were as follows: CGamF, 6.4; C-L-F, 5.0; CamF, 6.5; and UDCamF, 7.4. The absorbance of NBD compounds was determined at 478 nm. Extinction coefficients (in units of 10^4 mol/L^{-1} cm^{-1}) were as follows: C-L-NBD, 2.1; and UDC-L-NBD, 2.5.

To determine hepatic uptake (first-pass fractional extraction), 3-mL samples of influent and effluent were taken, and their absorbance measured to give the influent substrate concentration (C_i) and the effluent substrate concentration (C_o).

Biliary recovery of radiolabeled bile acids was determined by measuring biliary radioactivity divided by the specific activity of the infused compound. To do this, 10-µL bile aliquots were mixed with 8 mL of a toluene base scintillation fluid (ScintiVerse BD, Fisher Scientific, Fair Lawn, NJ). In the case of the perfusate, 1-mL samples of influent or effluent were added to the scintillation fluid. Radioactivity was then measured using a liquid scintillation counter (Beckman LS 3801, Beckman Instruments Inc., Fullerton, CA). Quenching was corrected for by an external standard ratio method.

Perfusate and bile volume was determined gravimetrically, assuming a density of 1.00 g/mL.

Data Analysis and Transport Equations. Data are expressed as mean $\pm$ SD. Where appropriate, differences between groups were tested for significance using the unpaired Student's t test or ANOVA. $P < .05$ was considered significant.

Net basolateral membrane transport (equivalent to clearance [$CL_{basolateral}$]) was calculated as the product of perfusate flow rate (Q) and fractional hepatic extraction (FE):

$$CL_{basolateral} = Q \times FE = Q \times [(C_i - C_o)/C_i]^{26}$$

For fluorescent bile acids, norUDCA, and fluorescein, the net extraction (percent) declined continuously with time, and the value obtained at the end of the 15-minute perfusion ("approach to steady-state") was used for this calculation. (This value is a minimal figure for $CL_{basolateral}$.) For conjugated bile acids, the percent extraction remained constant with time, and the mean percent net extraction value was used.

Intrinsic hepatic clearance ($CL_{intrinsic}$) is defined as the maximum possible hepatic clearance, i.e., hepatic clearance when flow is not rate-limiting (or removal rate adjusted for the decreasing concentration along the hepatic lobule). This parameter, which indicates the intrinsic ability of the liver to remove a test compound from the perfusate, was calculated using the equation:

$$CL_{intrinsic} = -Q \times \ln(1 - FE)^{27}$$

Cumulative basolateral regurgitation was obtained by measuring

TABLE 1. Fluxes of Fluorescent Bile Acids and Natural Bile Acids at the Basolateral and Canalicular Poles of the Hepatocyte: Bile Acids With a Cholyl-Moiety

Compound Number of IPRL studies	CGamF 3	C-L-F 3	C-L-NBD 3	CamF 3	C-glycine 2	C-taurine 2
Basolateral (sinusoidal) pole						
Net FE T_3 (%)	85.4 ± 2.6	90.5 ± 1.6	97.4 ± 0.8	70.1 ± 6.5	94.6 ± 1.2	72.4 ± 0.4
Net FE T_{15} (%)	63.4 ± 4.7	82.8 ± 2.5	86.1 ± 1.8	51.7 ± 4.3	93.9 ± 1.4	71.1 ± 0.4
Maximal uptake rate* (nmol/g liver × min)	27.7 ± 1.8	32.0 ± 2.4	29.0 ± 1.7	26.6 ± 4.1	31.0 ± 1.6	25.0 ± 2.1
Cumulative uptake in 60 min (% infused)	75.5 ± 3.8	86.9 ± 2.6	89.3 ± 0.8	63.5 ± 6.6	94.0 ± 0.8	71.5 ± 0.2
$CL_{basolateral}$* (mL/g liver × min)	1.91 ± 0.27	2.81 ± 0.27	2.53 ± 0.24	1.49 ± 0.16	3.30 ± 0.21	2.31 ± 0.25
$CL_{intrinsic}$ (mL/g liver × min)	3.10 ± 0.55	6.08 ± 0.80	5.79 ± 0.73	2.35 ± 0.56	9.05 ± 0.57	4.04 ± 0.43
Regurgitation (T_{15}-T_{60}) (% uptake)	2.23 ± 0.55	1.08 ± 0.19	4.05 ± 0.14	4.70 ± 0.20	0.75 ± 0.35	6.25 ± 1.34
Apical (canalicular) pole						
Maximal output rate* (nmol/g liver × min)	14.1 ± 1.3	23.1 ± 1.6	13.3 ± 1.5	7.7 ± 1.9	27.7 ± 1.0	22.7 ± 1.2
Cumulative recovery in 60 min (% uptake)	92.5 ± 5.2	83.1 ± 6.2	81.9 ± 1.2	74.4 ± 9.5	98.4 ± 1.9	98.1 ± 0.6
Fractional turnover rate of mass in liver (min^{-1})	0.35 ± 0.01	0.35 ± 0.01	0.25 ± 0.10	0.18 ± 0.07	0.75 ± 0.11	1.12 ± 0.04
Mass retained T_{60}* (nmol/g liver)	24.4 ± 18.9	84.5 ± 29.5	69.3 ± 11.6	66.2 ± 28.9	3.6 ± 4.7	7.7 ± 3.1
Canalicular output (max)/basolateral uptake (max)*	0.53	0.71	0.45	0.29	0.91	0.91

NOTE. Data are mean ± SD.

Abbreviations: FE, fractional extraction; T_x, perfusion time, where x = time of observation in minutes; CL, clearance.

*These parameters are strictly determined by the infused load (24-36 nmol/g liver × min × 15 min).

compound recovery in the effluent for samples obtained between 15 and 60 minutes. The value was expressed as percent of the amount taken up by the liver.

Hepatic sequestration was defined as the difference between net uptake (during the first 15 minutes of perfusion) and cumulative biliary recovery during the entire 60-minute perfusion. In addition, a plot was made showing biliary recovery as a function of the amount of substrate present in the liver. Such a curve is termed the biliary output/hepatic content relationship curve. The intercept of the curve with the horizontal x axis indicates the amount of compound in nanomoles per gram of liver sequestrated by the liver after 60 minutes of biliary excretion (SigmaPlot 2.0, Jandel Scientific, San Rafael, CA).

The value for maximum canalicular transport was assumed to be identical to maximum biliary recovery. A value defined as fractional turnover rate of the mass in the liver (in minutes⁻¹) was obtained by determining the rate of canalicular transport in relation to the decline in hepatic content (Δ biliary output/Δ hepatic content). This was performed by linear regression using the final part of the curve describing biliary recovery in relation to liver content (SigmaPlot 2.0).

The choleretic activity of the infused compounds was not calculated in these experiments, because the choleresis induced by infusing bile acids at the rate used in the present study is quite small relative to bile acid-independent flow. Values obtained under near-steady-state conditions in the anesthetized biliary fistula rat have been reported previously.[15]

RESULTS

Overall Hepatic Transport. During the initial few minutes of perfusion, all fluorescent bile acids were efficiently extracted from the albumin-free perfusate with net fractional hepatic extraction values at 3 minutes ranging from 70% to 97% (Tables 1 and 2). For natural conjugated bile acids, the net fractional extraction of cholylglycine was 95%, and that of

TABLE 2. Fluxes of Fluorescent Bile Acids, Natural Bile Acids and Fluorescein at the Basolateral and Canalicular Poles of the Hepatocyte: Bile Acids with a UDC-Moiety

Compound Number of IPRL studies	UDCamF 3	UDC-L-NBD 3	UDC-glycine 2	norUDCA† 2	Fluorescein‡ 3
Basolateral (sinusoidal) pole					
Net FE T_3 (%)	95.3 ± 1.1	92.2 ± 3.5	99.7 ± 0.1	95.9 ± 2.1	68.4 ± 9.9
Net FE T_{15} (%)	85.1 ± 4.0	85.8 ± 2.5	99.6 ± 0.1	72.8 ± 1.6	53.5 ± 5.7
Maximal uptake rate* (nmol/g liver × min)	30.9 ± 2.7	31.1 ± 1.5	35.1 ± 2.4	28.3 ± 0.4	21.7 ± 4.1
Cumulative uptake in 60 min (% infused)	89.8 ± 2.6	87.2 ± 3.0	99.6 ± 0.1	81.9 ± 3.1	58.8 ± 8.0
$CL_{basolateral}$* (mL/g liver × min)	2.60 ± 0.15	2.73 ± 0.10	3.61 ± 0.19	2.12 ± 0.10	1.65 ± 0.44
$CL_{intrinsic}$ (mL/g liver × min)	5.71 ± 1.13	6.75 ± 0.39	21.38 ± 0.39	3.82 ± 0.28	2.37 ± 0.57
Regurgitation (T_{15}-T_{60}) (% uptake)	0.70 ± 0.10	2.55 ± 0.35	0.39 ± 0.41	5.62 ± 0.04§	6.30 ± 0.56
Apical (canalicular) pole					
Maximal output rate* (nmol/g liver × min)	9.4 ± 2.3	1.1 ± 0.3	31.6 ± 1.3	—	8.5 ± 3.2
Cumulative recovery in 60 min (% uptake)	67.7 ± 3.0	12.2 ± 3.7	96.7 ± 3.8	77.6 ± 4.9	59.3 ± 20.3
Fractional turnover rate of mass in liver (min^{-1})	0.09 ± 0.02	0.01 ± 0.01	1.01 ± 0.02	—	0.13 ± 0.01
Mass retained T_{60}* (nmol/g liver)	125.7 ± 37.5	341.1 ± 31.3	15.5 ± 20.4	83.7 ± 20.4	113.7 ± 49.9
Canalicular output (max)/basolateral uptake (max)*	0.31	0.04	0.91	—	≥0.38

NOTE. Data are mean ± SD.

Abbreviations: FE, fractional extraction; T_x, perfusion time, where x = time of observation in minutes; CL, clearance.

*These parameters are strictly determined by the infused load (24-36 nmol/g liver × min × 15 min).

†Because norUDCA undergoes cholehepatic shunting, values for canalicular output and fractional turnover rate are not given.

‡For fluorescein, if cholehepatic shunting occurs, canalicular output will be underestimated and regurgitation will include fluxes from cholangiocytes.

§For norUDCA, regurgitation will include fluxes from cholangiocytes.

UDC-glycine was >99%, both values significantly greater than that of cholyltaurine (72%; $P < .005$). However, during the first minute of perfusion, cholyltaurine uptake exceeded that of cholylglycine. Fluorescein, whose uptake by the hepatocyte is considered to occur passively without involvement of a carrier,[28] had the lowest first-pass extraction of any of the compounds (68%). Fractional extraction at 15 minutes declined for all fluorescent bile acids, mostly because of regurgitation. In contrast, the fractional extraction of natural conjugated bile acids did not decrease with time (see below). Basolateral uptake efficiency can be expressed as intrinsic clearance that relates the uptake rate to the logarithmic concentration of the bile acid in the perfusate. Values for the intrinsic clearances of fluorescent bile acids ranged from 2.4 to 6.8 (mL/g liver · min), and a similar value was obtained for cholyltaurine (4.0). The intrinsic clearance of cholylglycine (9.1) and UDC-glycine (21.4) were greater than those of all fluorescent bile acids and cholyltaurine. Values for basolateral membrane fluxes are summarized in Tables 1 and 2.

All compounds were secreted into bile, but the rate of canalicular secretion and the completeness of biliary recovery varied widely (Tables 1 and 2). The time course of hepatic uptake and of biliary output for bile acids having a cholyl moiety are shown in the *left panels* of Figs. 2 and 3. The time course of hepatic uptake and biliary output of bile acids having a UDC moiety are shown in the *left panel* of Fig. 4. The time course of hepatic uptake and biliary output of norUDCA and fluorescein are shown in the *left panel* of Fig. 5. The relationships between biliary output and mass in the liver are shown on the *right panels* in Figs. 2, 3, 4, and 5.

Among the fluorescent bile acids, biliary recovery, expressed as percent of total hepatic uptake, differed considerably between compounds, ranging from 93% to 12%. The most complete biliary recovery was observed for CGamF (92%) and C-L-F (83%); the least was for UDC-L-NBD (12%). (Cholehepatic shunting cannot contribute to incomplete biliary recovery, because these fluorescent bile acids have been shown previously to have little passive membrane permeability.[10,13,15])

The biliary recoveries of cholylglycine, cholyltaurine, and UDC-glycine, natural conjugated bile acids, were essentially complete, exceeding 97%. ($P < .05$ for recovery of fluorescent bile acids vs. natural conjugated bile acids). NorUDCA, which undergoes cholehepatic shunting,[17,18] had the lowest recovery of any of the natural bile acids (78%), as anticipated. The recovery of fluorescein (as such, and as its glucuronide[15]) was also incomplete, averaging 59%. The fractional turnover rate (in min^{-1}) of the mass of bile acid in the liver ranged from 0.01 to 0.35; these values were markedly below those of the natural conjugated bile acids, which ranged from 0.75 to 1.12, indicating that the fluorescent bile acids were retained much longer in the hepatocyte for a given apparent concentration.

Time-Dependent Effects on Net Fractional Extraction. The time course of percent net fractional extraction is shown in Fig. 6. Net extraction decreased with time for all fluorescent bile acids, indicating reflux across the basolateral membrane into the perfusate and/or decreased hepatic uptake. There was a close relationship between cumulative basolateral regurgitation measured directly and the time-dependent decrease in hepatic fractional extraction ($r = .87$; data not shown), indicating that much of the continuing decrease in hepatic extraction could be attributed to regurgitation. The rate of
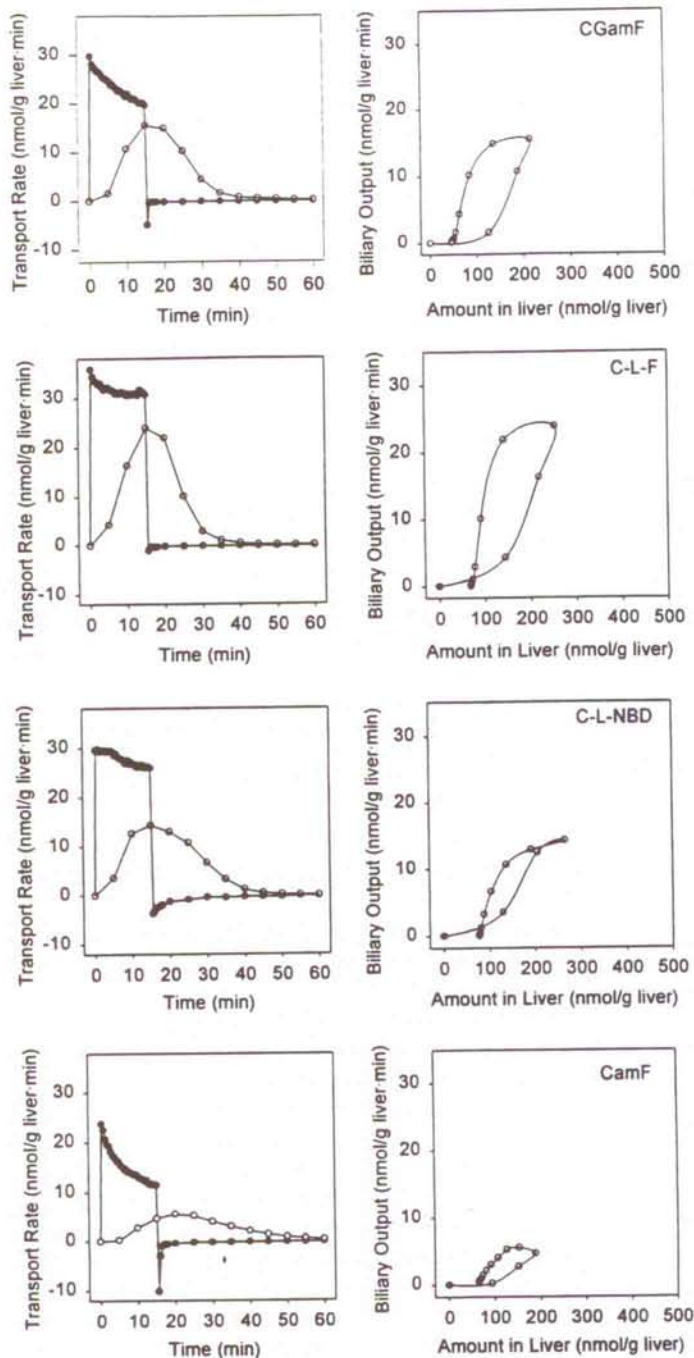

FIG. 2. Time-course of hepatic uptake (●) and biliary output (○) for fluorescent bile acids with a cholyl (C) moiety (*left panel*). Relationship between biliary output (vertical axis) and mass in the liver (horizontal axis) for these bile acids (*right panel*). For explanations of abbreviations and chemical structures, see Fig. 1.

decline in the percent net extraction decreased with time, indicating that steady-state conditions were being approached. A similar decrease in the uptake with time was observed for norUDCA and fluorescein, raising the possibility that fluorescein also undergoes a cholehepatic circulation. Only unconjugated fluorescein was present in the effluent (based on thin-layer chromatography), indicating that fluorescein glucuronide did not reflux.

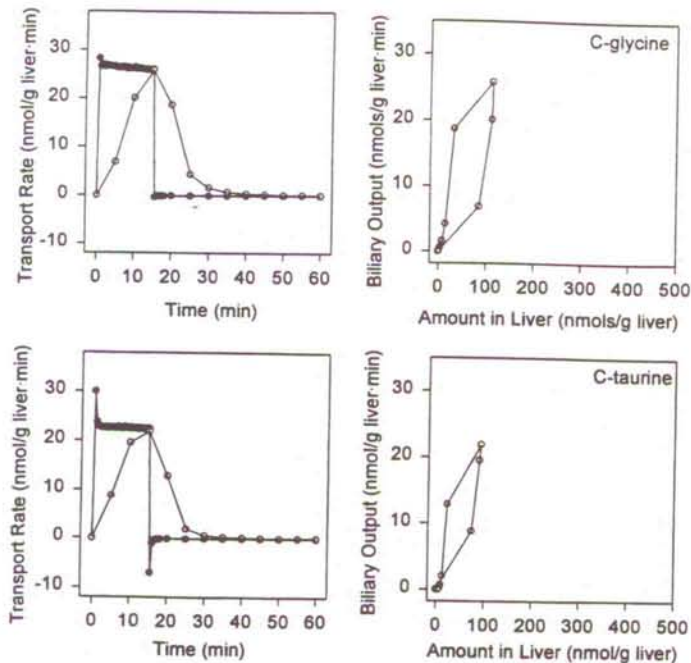In contrast, net extraction of cholylglycine, cholyltaurine,

FIG. 3. Time-course of hepatic uptake (●) and biliary output (○) for natural bile acid amidates (*left panel*). Relationship between biliary output (vertical axis) and mass in the liver (horizontal axis) for these bile acids (*right panel*). C-glycine, cholylglycine; C-taurine, cholyltaurine.

and UDC-glycine remained unchanged during the 15-minute perfusion (Fig. 6).

Mobilization of Sequestered Fluorescent Bile Acids. The addition of DBcAMP to the perfusate caused a 75% decrease in hepatobiliary retention of UDCamF. In contrast, coinfusion of cholyltaurine had no effect, and UDC-taurine caused only a limited mobilization of this fluorescent bile acid (Fig. 7).

In contrast, addition of DBcAMP to the perfusate had no effect on biliary secretion of sequestered UDC-L-NBD (Fig. 8). However, coinfusion of cholyltaurine or UDC-taurine caused a marked mobilization of the sequestered UDC-L-NBD, with biliary output increasing sixfold, from 12% to 60%–70%. A similar mobilization was induced by deoxycholyltaurine (data not shown), indicating that this effect was shared by at least three taurine-conjugated bile acids varying considerably in hydrophobicity. UDC-L-NBD was also efficiently mobilized by the addition of cholyltaurine to the perfusate well after sequestration had occurred (data not shown), indicating that UDC-L-NBD can be transported from its sequestration site(s) and by canalicular transporter(s).

DISCUSSION

These results extend considerably our previous observations on hepatobiliary transport of fluorescent bile acids that were obtained in the intact animal.[15] In the isolated perfused liver, all fluorescent bile acids had efficient net hepatic extraction at the infusion rate employed in these studies (≈0.04 μmol/g liver · min). (This rate is about one tenth the physiological transport rate of conjugated bile acids.[29]) Within a few minutes, net extraction of fluorescent bile acids decreased, in part because of regurgitation.

Biliary recovery varied widely among the six fluorescent bile acids. For all compounds, sequestration in the hepatocyte occurred, as indicated by a complete cessation of biliary

output despite the compound being present in the liver at high apparent concentrations. The much greater sequestration of UDC-L-NBD as compared with that of C-L-NBD was not astonishing, because a lower recovery of UDC-L-NBD than of C-L-NBD was observed in our previous study in the biliary fistula rat.[15] The natural bile acid conjugates used in this study (cholylglycine, cholyltaurine, and UDC-glycine) did not exhibit sequestration. For fluorescent bile acids, the flux rate across the basolateral pole exceeded that across the canalicular pole, in agreement with previous studies with cholephilic anions such as sulfobromophthalein.[30-32] For natural conjugated bile acids, at the infusion rate employed, the maximal transport rates across the basolateral membrane and the canalicular membrane were similar.

A second finding was that sequestered UDCamF could be mobilized by a cAMP precursor, but not by conjugated bile acids, whereas UDC-L-NBD could be mobilized by conjugated bile acids, but not by the cAMP agonist. Thus, two types of intracellular trapping were observed.

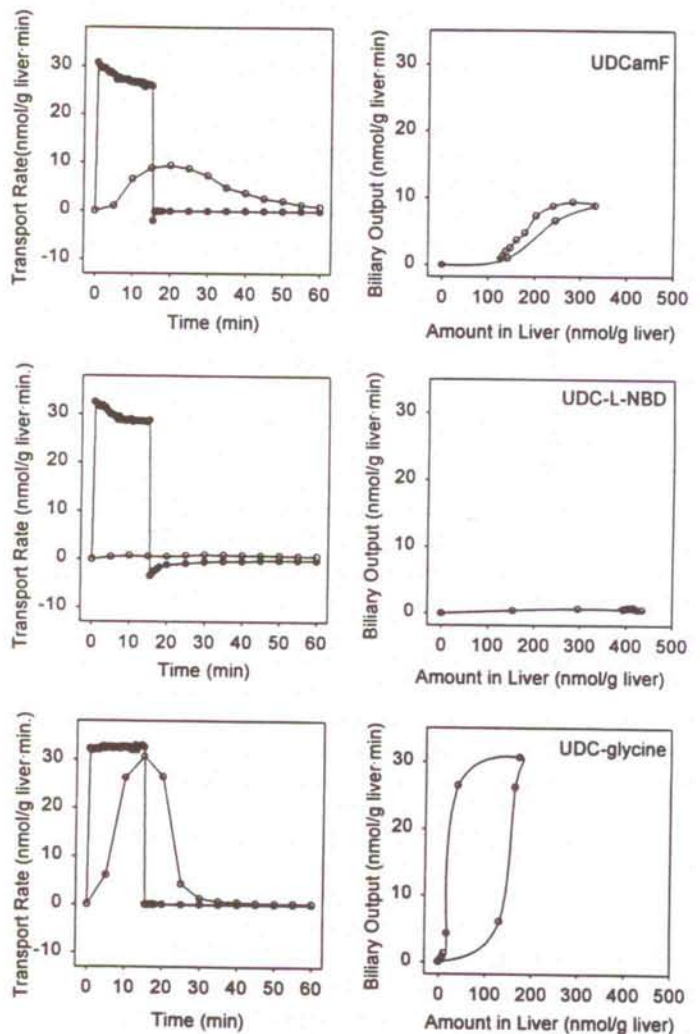*Decrease in Net Fractional Hepatic Extraction.* The simplest explanation for the time-dependent decrease in the net

FIG. 4. Time-course of hepatic uptake (●) and biliary output (○) for bile acids with a UDC moiety (*left panel*). Relationship between biliary output (vertical axis) and mass in the liver (horizontal axis) for these bile acids (*right panel*). For explanations of abbreviations and chemical structures, see Fig. 1.
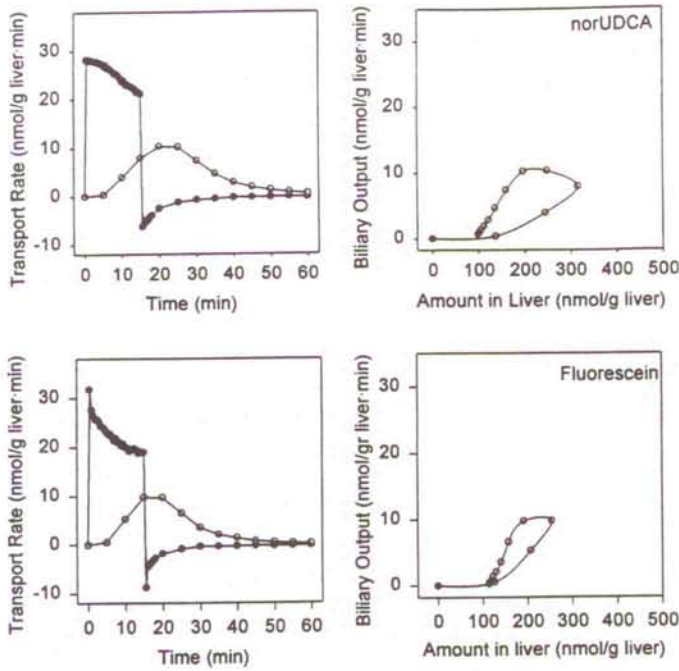
FIG. 5. Time-course of hepatic uptake (●) and biliary output (○) for norUDCA and fluorescein (*left panel*). Relationship between biliary output (vertical axis) and mass in the liver (horizontal axis) for these compounds (*right panel*).

fractional extraction of the fluorescent bile acids is that an increased intracellular concentration caused reflux, as evidenced by the high correlation between reflux measured directly and the time-dependent decrease in net fractional extraction. Reflux is presumed to have occurred via the bidirectional basolateral sodium-independent organic anion transporter termed *oatp*.[33] Additional participation of *ntcp*, a sodium-dependent transporter for conjugated bile acids,[34] cannot be excluded. Regurgitation, presumably carrier-mediated, has also been shown for charged organic anions such as dibromosulfophthalein.[35] The absence of reflux of cholylglycine (and UDC-glycine) is likely to be explained by efficient binding to cytosolic proteins, because cholylglycine is known to be a substrate for *oatp*.[33] Reflux of cholyltaurine, as assessed by cumulative basolateral regurgitation, was 10

times greater than that of cholylglycine, explaining its lower hepatic extraction (72%). Nonetheless, Takikawa et al.[36] showed that regurgitation of cholylglycine can be induced in the isolated perfused liver by coinfusing indomethacin, which is known to compete against bile acids for intracellular binding sites. If this argument is correct, the fluorescent bile acids were less tightly bound intracellularly than the glycine-conjugated bile acids. Of course, molecules that regurgitate from a periportal hepatocyte will be taken up by the pericentral cells; thus, the process of uptake and regurgitation continues along the entire hepatic lobule.[35]

Regurgitation that is non-carrier-mediated can also explain in part the similar time-dependent decrease in net fractional extraction of fluorescein and norUDCA, because both fluorescein[28] and norUDCA (based on the properties of its epimer norchenodeoxycholic acid[37]) are thought to be highly permeable to cell membranes and should regurgitate from the hepatocyte when their intracellular (unbound) concentration exceeds that in the perfusate. Such regurgitation has been shown previously for nordeoxycholic acid,[38] a molecule that should be handled by the basolateral membrane of the hepatocyte in a manner similar to that of norUDCA. For both norUDCA and fluorescein, cholehepatic shunting is also likely to have occurred, and this will contribute to the observed time-dependent decrease in fractional extraction.[38]

In the present study, the first-pass fractional extraction of cholyltaurine after the first minute (72%) was in good agreement with published values for the isolated perfused rat liver.[39] After the first minute, the fractional extraction of cholylglycine was consistently and markedly higher (94%) than that of cholyltaurine; thus, these two amidates of cholic acid with rather similar side-chain topology do not have identical net uptake rates by the liver. Aldini et al.,[40] using an indicator dilution method and a "naive" liver, reported that cholyltaurine had a greater percent net extraction than cholylglycine. Our results confirm the report of Aldini et al. in that greater uptake of cholyltaurine occurred for the first two samples, i.e., samples obtained at 0.5 and 1.0 minutes (three experiments). It appears that cholyltaurine disappearance from the perfusate results from not only transport into the hepatocyte, but also from binding to high-affinity sites on the basolateral membrane of the hepatocyte (or other cell membranes in the sinusoid). At any rate, our data indicate
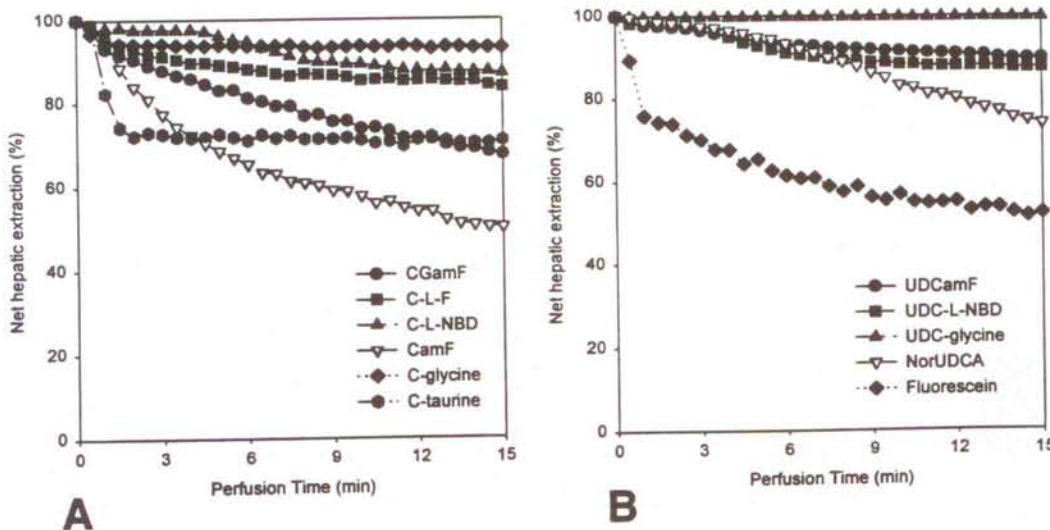

FIG. 6. Time-course of percent net hepatic extraction of bile acids and fluorescein from the perfusate. (A) Fluorescent and natural bile acids with a cholyl (C) moiety. (B) Fluorescent and natural bile with a UDC moiety, norUDCA and fluorescein. For explanations of abbreviations and chemical structure of fluorescent bile acids, see Fig. 1. C-glycine, cholylglycine; C-taurine, cholyltaurine.

FIG. 7. Effects of addition of DB-cAMP to the perfusate or coinfusion of natural bile acids (cholyltaurine or UDC-taurine) on hepatobiliary sequestration and mobilization of UDCamF. (A) Hepatobiliary sequestration after 45 minutes substrate-free perfusion (mean ± SD; $n \geq 2$ for all variables). (B) Biliary output as a function of the amount of substrate present in the liver with and without the addition of DBcAMP to the perfusate (representative experiment). *Vertical arrows* indicate amount sequestered in liver without (basal) (*right arrow*) and with (*left arrow*) addition of DBcAMP. *A significant difference between recovery without and with added DBcAMP ($P < .05$).
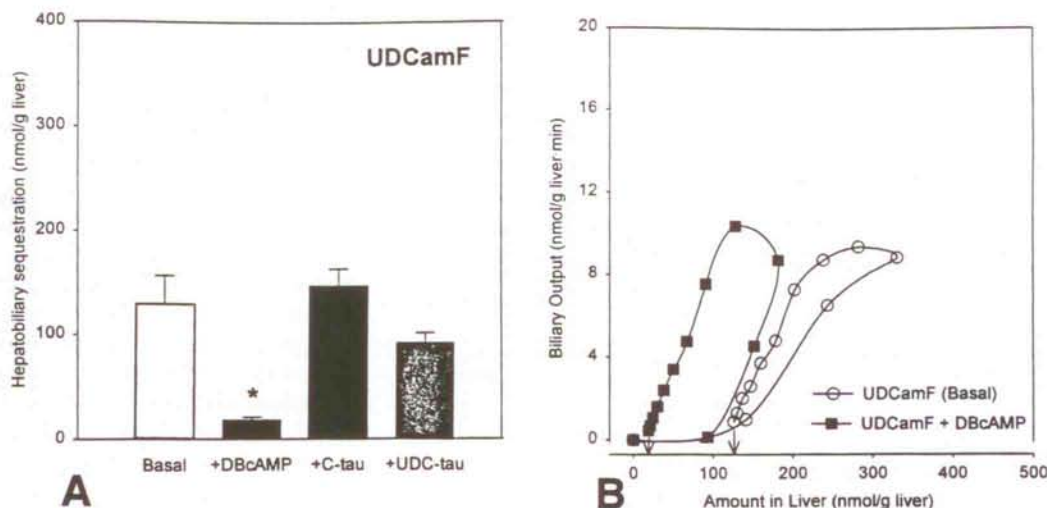
that as steady-state conditions are approached, net uptake of cholyltaurine is considerably less efficient than that of cholylglycine because of regurgitation of cholyltaurine.

Mechanisms of Sequestration of Fluorescent Bile Acids. Experiments at the perfused organ level do not provide direct information on the nature of hepatocyte sequestration. Possibilities include binding to cytosolic proteins and/or organelles such as microsomes, mitochondria, pericanalicular vesicles, and nuclei. Binding may be to the surface of organelles or may involve carrier-mediated transport into or out of their interior.

The mobilization of UDCamF by a cAMP precursor can be explained by the cAMP causing fusion of a vesicular compartment with the canalicular membrane, thus making the UDCamF in the vesicle available for transport into bile. Evidence for such mobilization of latent canalicular vesicles has been provided in an elegant study by Boyer and Soroka.[11]

The mobilization of UDC-L-NBD by coinfusion of conjugated bile acids can be explained by the conjugated bile acids either displacing the bound fluorescent bile acid from cytosolic proteins or organelle surfaces, or competing for transport by a carrier present in organelles, or both. Several groups have provided evidence for a transport system for conjugated bile acids being present in the smooth endoplasmic reticulum[41,42]; a candidate for such a transporter is epoxide hydrolase.[43] The sites of sequestration of UDCamF and UDC-L-NBD appear to have a finite storage capacity, because both of these compounds were secreted into bile at a constant rate when they were infused intravenously for a 90-minute period.[15] In addition, UDC-L-NBD was mobilized by the addition of cholyltaurine to the perfusate 45 minutes after sequestration had occurred.

Figure 9 is a scheme for the transport of fluorescent bile acids by the hepatocyte using the conventions of compartmental analysis that can provide an explanation for the two types of mobilization responses.

The mobilization of sequestered fluorescent bile acids by the coinfusion of conjugated bile acids is analogous to the mobilization of cholestatic bile acids such as chenodeoxycholyl-taurine or lithocholyltaurine (taurolithocholate) by the coinfusion of bile acids such as UDC-taurine.[44-47] If the sequestered fluorescent bile acids are surrogates for endogenous cytotoxic bile acids, our results suggest that the cytoprotective effect of UDCA observed, for example, with isolated hepatocytes (see Benz[48]), can be explained by UDCA preventing sequestration of cytotoxic bile acids in organelles. Such a mechanism of cytoprotection might be termed "organelle blockade." Conjugated bile acids also stimulated mobilization of previously sequestered flourescent bile acids.

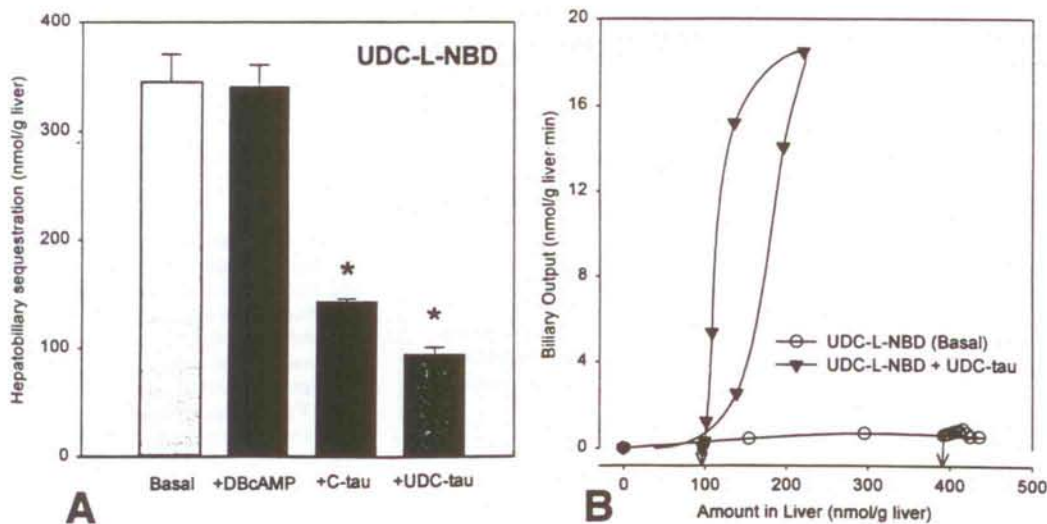The term sequestration has been used with several different

FIG. 8. Effects of addition of DBcAMP to the perfusate and coinfusion of natural bile acids (cholyltaurine and UDC-taurine) on hepatobiliary sequestration and mobilization of UDC-L-NBD. (A) Hepatobiliary sequestration after 45 minutes substrate-free perfusion (mean ± SD; $n \geq 2$ for all variables). (B) Biliary output as a function of the amount of substrate present in the liver with and without coinfusion of UDC-taurine. A representative experiment is shown. *A significant difference between recovery without and with added bile acid amidates ($P < .005$).
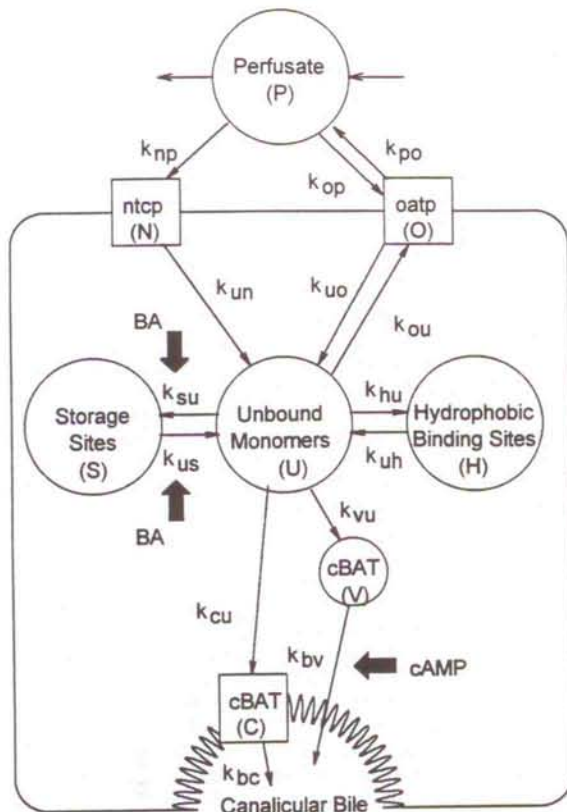
Perfusate
(P)

k_{np} k_{po}

k_{op}

ntcp
(N)   oatp
(O)

k_{un} k_{uo}

k_{ou}

BA

k_{su} Unbound
Monomers
(U) k_{hu} Hydrophobic
Binding Sites
(H)

Storage
Sites
(S) k_{us} k_{uh}

BA

k_{vu}

cBAT
(V)

k_{cu}

k_{bv} cAMP

cBAT
(C)

k_{bc}
Canalicular Bile

FIG. 9. Compartmental model for hepatocyte transport of fluorescent bile acids and mobilization by coinfusion of conjugated bile acids or a cAMP precursor. *Arrows* indicate transfer coefficients. *Circles* denote intracellular pools; *squares* denote transporters without appreciable mass. The term "binding sites" denotes reversible binding with rapid on and off rates. The term "storage sites" denotes bound ("sequestered") molecules that are released slowly in relation to the time of the experiment; it does not denote any particular organelle. Mobilization of sequestered UDC-L-NBD by infusion of conjugated bile acids (*vertical arrows*, BA) is most easily explained by inhibition of transport into, or by stimulation of transport out of, storage sites. Mobilization of sequestered UDCamf by DBcAMP (*horizontal arrow*, cAMP) is most easily explained by the induced targeting to the canalicular membrane of vesicles containing cBAT (the canalicular bile acid transporter). Targeting is indicated by the transfer coefficient k_{vb}, rather than by translocation of the vesicles, for convenience.

meanings in the isolated perfused liver. It has long been used to describe the apparently irreversible binding of lipophilic molecules by the isolated perfused liver.[49] Matheny et al.[50] noted that a tracer dose of morphine previously taken up by the isolated liver would regurgitate when a much larger dose of morphine was added to the perfusate; they used the term "sequestered" for the mobilizable pool of morphine. Sequestration of cationic compounds in acidic vesicles is well documented.[51]

Experimental Design. The use of a 15-minute infusion allowed uptake, storage, and secretion events to be defined, as the steady-state was approached. The late Carl Goresky initially proposed the use of the indicator dilution technique with a "naive" liver,[52] but subsequently he recommended measurement of uptake (and regurgitation) using a tracer dose superimposed on a steady-state infusion,[53] the design thus being a traditional pulse-chase experiment. The technique reported here appears to provide information that is lacking in the steady-state design, because it permits the time course of hepatic sequestration to be defined. The desirability

of studying drug transport using an "approach to steady-state" has also been supported in a detailed study of lidocaine metabolism in the perfused liver by Saville et al.[54]

Evidence Against Cholehepatic Shunting of Conjugated Bile Acids. NorUDCA was included among the natural bile acids, because multiple lines of evidence indicate that this molecule undergoes cholehepatic shunting in the rat.[17,18] Two groups have independently reported that the apical bile salt cotransporter is not only present in the ileal enterocyte and renal tubular epithelial cell, but also in cholangiocytes.[55,56] This observation has raised the possibility that not only unconjugated dihydroxy bile acids, but even conjugated bile acids, undergo cholehepatic shunting. We have previously reported evidence that C-L-NBD is a substrate for the apical bile salt transporter, based on its absorption from the perfused rat ileum.[15] In contrast, CGamF was not a substrate for this transporter, because essentially no absorption from the perfused ileum was detected.[15] Our observation that these two fluorescent bile acid molecules have similar biliary output/ hepatobiliary content curves, neither of which resembles that of norUDCA, suggests to us that it is unlikely that there is an appreciable flux of conjugated bile acids via the cholehepatic circulation in the portally perfused rat liver. Our data certainly do not exclude the possibility of a small flux of conjugated bile acids through the cholangiocyte mediated by this transporter; such transport might act to potentiate secretin-mediated bicarbonate secretion by the cholangiocyte, as proposed by Alpini et al.[57]

CONCLUSIONS

In summary, fluorescent bile acids are efficiently taken up by the isolated rat liver perfused in nonrecirculating mode, but recovered to a variable extent in bile because of hepatic sequestration. The molecules whose properties most resemble those of cholylglycine and cholyltaurine are CGamF and C-L-F. Two modes of hepatic sequestration were identified phenomenologically, based on the types of molecules used to mobilize stored molecules. Further insight into the sites of hepatic sequestration and the mechanism(s) of mobilization will require techniques for determining the intracellular location of these molecules such as confocal fluorescence microscopy.[12]

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