Physicochemical and physiological properties of 5α-cyprinol sulfate, the toxic bile salt of cyprinid fish

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Abstract 5α-Cyprinol sulfate was isolated from bile of the Asiatic carp, *Cyprinus carpio*. 5α-Cyprinol sulfate was surface active and formed micelles; its critical micellization concentration (CMC) in 0.15 M Na^+ using the maximum bubble pressure device was 1.5 mM; by dye solubilization, its CMC was ~4 mM. At concentrations >1 mM, 5α-cyprinol sulfate solubilized monooleylglycerol efficiently (2.1 molecules per mol micellar bile salt). When infused intravenously into the anesthetized rat, 5α-cyprinol sulfate was hemolytic, cholestatic, and toxic. In the isolated rat liver, it underwent little biotransformation and was poorly transported ($T_{max} \cong 0.5$ μmol/min/kg) as compared with taurocholate. 5α-Cyprinol, its bile alcohol moiety, was oxidized to its corresponding C_{27} bile acid and to allocholic acid (the latter was then conjugated with taurine); these metabolites were efficiently transported. 5α-Cyprinol sulfate inhibited taurocholate uptake in COS-7 cells transfected with rat *asbt*, the apical bile salt transporter of the ileal enterocyte. 5α-Cyprinol had limited aqueous solubility (0.3 mM) and was poorly absorbed from the perfused rat jejunum or ileum. Sampling of carp intestinal content indicated that 5α-cyprinol sulfate was present at micellar concentrations, and that it did not undergo hydrolysis during intestinal transit. These studies indicate that 5α-cyprinol sulfate is an excellent digestive detergent and suggest that a micellar phase is present during digestion in cyprinid fish.—Goto, T., F. Holzinger, L. R. Hagey, C. Cerrè, H-T. Ton-Nu, C. D. Schteingart, J. H. Steinbach, B. L. Shneider, and A. F. Hofmann. **Physicochemical and physiological properties of 5α-cyprinol sulfate, the toxic bile salt of cyprinid fish. *J. Lipid Res.* 2003. 44: 1643–1651.**

Supplementary key words *Cyprinus carpio* • bile acids • micelles • bacterial deconjugation • fat digestion • fat absorption • hepatic transport • cholestasis • intestinal absorption • solubilization

In vertebrates, cholesterol is eliminated by conversion to water-soluble amphipathic, functional molecules called bile salts. Bile salts can be divided into three classes based on side-chain structure: C_{27} bile alcohols, C_{27} bile acids, and C_{24} bile acids (1). After their biosynthesis from cholesterol, bile alcohols and bile acids undergo "conjugation," a biotransformation step that renders them water soluble and membrane impermeable at physiological pH. Bile alcohols are conjugated by esterification of the terminal C-27 hydroxy group with sulfate, whereas bile acids are usually conjugated by N-acyl amidation of the terminal C-27 or C-24 carboxyl group with taurine or glycine (2, 3).

The occurrence of C_{27} bile alcohol sulfates is widespread in nature. They are the dominant bile salts of ancient mammalian species (elephant, manatee, hyrax, and rhinoceros) (4). They are also the major biliary surfactants present in cartilaginous fish (sharks, rays, and skates), herbivorous bony fish (carp, arapima, and angelfish), and in some amphibians (salamanders and frogs) (3, 5).

One of the common bile alcohols is 5α-cyprinol, a molecule with five hydroxy groups that was originally isolated from the bile of *Cyprinus carpio*, the Asiatic carp. Cyprinol was shown to have hydroxy groups at C-3, C-7, C-12, C-26, and C-27, based on the work of Hoshita, Magayoshi, and Kazuno (6) and Anderson, Briggs, and Haslewood (7). Confirmation of the structure of the sulfate ester of 5α-cyprinol by proton and ^{13}C-NMR as well as mass spectrometry (MS) has been reported by Asakawa et al. (8)[7]. The A/B ring juncture of cyprinol is 5α (A/B *trans*), whereas the structure of most C_{27} and C_{24} bile acids is 5β (A/B *cis*). It has become customary to add a 5α prefix to cyprinol to indicate clearly its 5α-A/B *trans* juncture, and thus distinguish it from 5β-cyprinol (A/B *cis*), which is present in other fish, such as the sturgeon (9). The structure of 5α-cyprinol sulfate is shown in **Fig. 1**.

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[7] Chemical Abstracts has assigned the registry number 15066-41-8 to 5α-cyprinol sulfate. Its index name is Cholestane-3,7,12,26,27-pentol, hydrogen sulfate, $(3\alpha,5\alpha,7\alpha,12\alpha)^-$. The assignment of the sulfate to C-27 versus C-26 is arbitrary.

Most natural bile acids are amphipathic, possessing a hydrophilic side and a hydrophobic side (10). The amphipathic structure of bile acids is responsible for their chief physiological function, which is to enhance absorption of dietary lipids. The C_{24} bile acids readily form mixed micelles with fatty acids and monoglycerides, and such mixed micelles can in turn solubilize fat-soluble vitamins. Such solubilization greatly enhances diffusion of insoluble lipids to the enterocyte brush border (11).

The physicochemical properties of C_{24} bile acids have been investigated extensively (12, 13), but few studies have examined the physicochemical properties of C_{27} bile acids and C_{27} bile alcohols. We hypothesized that the micelle-forming and solubilization properties of 5α-cyprinol sulfate should be similar to those of taurocholate, a molecule with a similar topology, as shown in **Fig. 2**, and performed studies to test this hypothesis. We also performed limited physiological studies on its ileal and hepatic transport in rodents because of its known toxicity for mammals (14–17), including humans [reviewed in (17)]. Finally, we examined some properties of 5α-cyprinol, the bile alcohol moiety of 5α-cyprinol sulfate, in order to define the possible in vivo significance of bacterial hydrolysis (deconjugation) of the ester bond linking sulfate to the bile alcohol.

METHODS

Isolation of 5α-cyprinol sulfate from carp bile

Gallbladders of *C. carpio* were obtained from a local fish market and an aquaculture facility (Loy Fisheries, Provo, UT) and stored in isopropanol. 5α-Cyprinol sulfate was isolated from the isopropanol-soluble extract of carp gallbladders. The extract was subjected to flash chromatography using a 30 × 5 cm column packed to 21 cm with silica gel, 40 μm (Flash Chrom Pack, J. T. Baker, Phillipsburg, NJ). The column was packed in chloroform-methanol (80:20; v/v). A highly concentrated isopropanol extract of carp bile was layered at the top of the column. A stepwise gradient of methanol in chloroform (80:20, 500 ml; 75:25, 500 ml; 70:30, 1,000 ml; 65:35, 500 ml) was used to elute the 5α-cyprinol sulfate. Fractions were examined by thin-layer chromatography (TLC) using a solvent system for conjugated bile acids (18). Fractions containing pure 5α-cyprinol sulfate (R_f 0.25) were pooled and taken to dryness on a rotary evaporator.

The structure of 5α-cyprinol sulfate (5α-cholestane-3α,7α,12α, 26,27-pentol-27-sulfate) was confirmed by proton magnetic resonance spectroscopy. Proton ^{1}H-NMR was carried out at 500 MHz in the Department of Chemistry, University of California, San Diego. The solvent was deuterated methanol, and chemical shifts are expressed in ppm relative to tetramethylsilane: 0.697 (s, 3H, Me-18), 0.793 (s, 3H, Me-19), 0.996 (d, 7.0 Hz, 3H, Me-21), 2.129 (tt, 12.5 Hz, 3.5 Hz, 1H, H-5), 3.540 ν and 3.566 ν (ABX, J_{ab} 11.0 Hz, J_{ax} 6.6 Hz, J_{bx} 5.6 Hz, 2H, H-27), 3.765 (d, 5.0 Hz, 1H, H-7), 3.928 (m, 1H, H-3), 3.960 (s, 1H, H-12), 3.985 ν and 4.015 ν (ABX, J_{ab} 9.5 Hz, J_{ax} 5.0 Hz, J_{bx} 6.5 Hz, 2H, H-26).

Preparation of 5α-cyprinol by solvolysis of 5α-cyprinol sulfate

5α-Cyprinol sulfate was precipitated from the isopropanol extract of carp gallbladders by the addition of several volumes of ethyl acetate. The precipitate (1.4 g) was dissolved in 2,2′-dimethoxypropane-1 N HCl (7:1; v/v) and maintained at 37°C for 12 h (19), the procedure resulting in complete solvolysis of the 5α-cyprinol sulfate. Water (200 ml) and chloroform-methanol (2:1, v/v) (800 ml) were added. The chloroform phase was evaporated to dryness, giving impure 5α-cyprinol. This was purified by silica gel column chromatography using chloroform-methanol, with stepwise increases in the proportion of methanol. Fractions were examined by TLC (18), and those containing pure 5α-cyprinol (R_f 0.66) were pooled to give 0.9 g of 5α-cyprinol that was pure by TLC. The molecular weight of 5α-cyprinol was confirmed by electrospray (ESI)-MS.

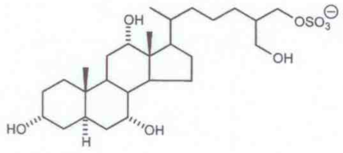

Fig. 1. Chemical structure of 5α-cyprinol sulfate.

Physicochemical properties of 5α-cyprinol sulfate and 5α-cyprinol

Determination of critical micellization temperature of 5α-cyprinol sulfate. The critical micellization temperature (CMT) (also termed Krafft point) is the temperature at which the solubility of the monomer reaches the critical micellization concentration (CMC). At this temperature, there is a phase change: insoluble, crystalline material dissolves and forms micelles. A 20 mM solution of 5α-cyprinol sulfate in water was kept at 4°C and observed daily for 4 days to see if 5α-cyprinol sulfate precipitated from solution.

Determination of ion product of the calcium salt of 5α-cyprinol sulfate. Bottles were prepared containing three bile salt concen-

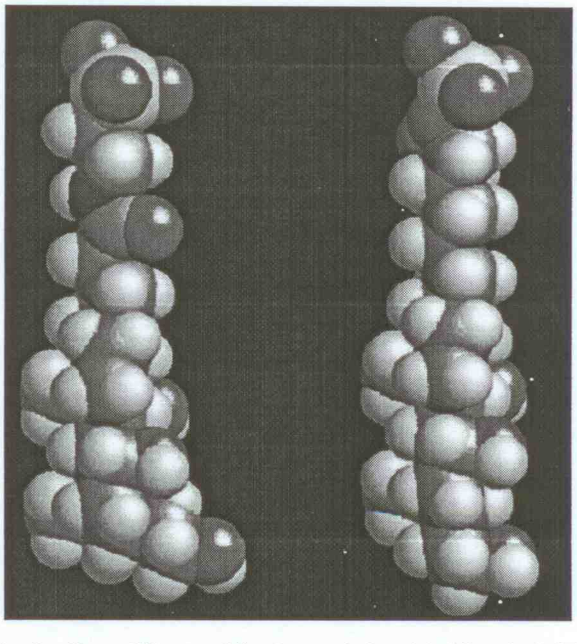

Fig. 2. Space-filling models of taurocholate (cholyltaurine), left panel, and 5α-cyprinol sulfate, right panel. Each molecule has a hydrophilic side and a hydrophobic side, and they are thus similar planar amphipaths. The *cis* A/B structure of taurocholate can be seen (bottom), as well as the nitrogen atom of taurine. The two molecules are quite similar in topology.

trations (3 mM, 5 mM, and 10 mM). For the 5 mM and 10 mM concentrations, calcium was added in increasing concentrations (0.2 M, 0.5 M, 1.0 M, 2.0 M, and 3.0 M). For the most-dilute bile salt concentration, calcium was added at the following concentrations: 0.1 M, 0.2 M, 0.3 M, 0.4 M, and 0.75 M. Solutions of the sodium salts of three other conjugated C_{24} bile acids (taurocholate, glycocholate, and glycodeoxycholate) were prepared similarly. All solutions/dispersions were kept at room temperature and examined daily in a dark room with a light beam to check for precipitation. The ion product was calculated as a $Ca^{2+} \times [\text{bile salt}]^{-2}$ with an activity coefficient of 0.3 used for Ca^{2+} (20). The midpoint between the bile salt concentration at which precipitation was not present and the lowest concentration at which it was present was used to calculate the ion product.

Determination of aqueous solubility of 5α-cyprinol. An excess of 5α-cyprinol was dispersed in distilled water and stirred intermittently for 1 week. The suspension was then centrifuged (2,000 *g* for 10 minutes) to sediment the insoluble 5α-cyprinol. One milligram of 5α-cholestane-3α,7α,12α, 24, 27-pentol dissolved in isopropanol was added to the supernatant. An aliquot was taken to dryness, converted to per-trimethylsilyl ethers using hexamethydilsilazane-trimethylchlorosilane-pyridine (2:1:10; v/v/v) (Tri-Sil Reagent, Pierce, Rockford, IL), and analyzed by gas chromatography (GC). The concentration of 5α-cyprinol was calculated from the peak ratio.

Determination of CMC of 5α-cyprinol sulfate. The CMC of 5α-cyprinol sulfate was determined in two ways. The first was based on the change in surface tension in relation to aqueous concentration using the maximum bubble pressure method. A commercial device (Sensadyne 6000 Tensiometer, Chem-Dyne Research Corp., Milwaukee, WI) was used. The device was calibrated with distilled water and methanol; a bubble frequency of 1 bubble/sec was used. Solutions of sodium taurochenodeoxycholate (chenodeoxycholyltaurine) and 5α-cyprinol sulfate were prepared (17 mM in bile salt, 137 mM NaCl), and the change in bubble pressure measured as the solutions were diluted progressively with 0.154 M NaCl (room temperature). The CMC was defined as the intersection of the two lines obtained by extrapolating the linear portions of the two curves obtained when surface tension was plotted against the logarithm of the bile salt concentration (10).

The CMC was also obtained by a dye solubilization technique using Orange OT (1-*O*-tolyl azo-2-naphthol), a water-insoluble, micelle-soluble dye. Solubilization of Orange OT occurs only when micelles are present, and the amount solubilized is directly proportional to the concentration of micelles. A line was drawn through the first three points obtained above the base line and extrapolated to the base line. The point of intersection of this line with the base line was defined as the CMC. Experiments were performed at the same time with sodium taurocholate to permit comparison of the CMC of a 5α-cyprinol sulfate with that of a bile acid having the same nuclear substituents. CMC values obtained by the maximum bubble pressure method and the dye solubilization technique are known to agree well (10).

Formation of mixed micelles by 5α-cyprinol sulfate with monooleylglycerol. Conjugated bile acid anions are known to associate cooperatively with amphiphilic, water-insoluble molecules such as monoglycerides, resulting in micelles being formed at concentrations well below the CMC of a simple bile acid solution.

The CMC of 5α-cyprinol sulfate in the presence of monooleylglycerol as well as its solubilizing capacity for this monoglyceride was determined by turbidometry as previously described (21).

Biological studies: metabolism and transport of 5α-cyprinol sulfate and 5α-cyprinol

Metabolism and transport of 5α-cyprinol sulfate and 5α-cyprinol in the isolated perfused rat liver (IPRL). Attempts to characterize hepatic transport and biotransformation of 5α-cyprinol sulfate in the anesthetized biliary fistula rat could not be performed because the intravenous infusion of 5α-cyprinol sulfate at the physiological rate of 1 μmol/min/kg (a rate that is physiological for hepatic transport of conjugated C_{24} bile acids in the rat) caused hemolysis, hemobilia, and death. Accordingly, all studies of hepatic transport and biotransformation were performed using the single-pass IPRL, as described in detail elsewhere (22). In this preparation, the liver is perfused with an electrolyte solution saturated with oxygen. Compounds were infused for 20 min at a physiological rate (for natural conjugated bile acids in the rat) of 1 μmol/min/kg animal body weight (about 25 nmol/g liver/min) or at 4 μmol/min/kg. Bile was collected in 5 min pools for 60 min.

Biliary secretion was determined gravimetrically, and bile salt output was estimated by the enzymatic method commonly used to measure bile acids (23). First-pass extraction was determined by measuring the bile salt concentration in the entering and leaving cannulae. Biotransformation of 5α-cyprinol sulfate and 5α-cyprinol was determined by a variety of chromatographic methods. These included TLC systems previously developed to characterize bile acid biotransformation (24), HPLC using a system previously described for separation of conjugated bile acids (25), GC-MS (26), and electrospray ionization (ESI)-MS. For GC-MS, unconjugated bile acids were isolated by ether extraction of acidified bile. GC-MS was performed after alkaline deconjugation (2N NaOH, 4 h at 130°C) to identify individual bile acids present in amidated form. GC-MS was also performed on eluates from TLC spots. ESI-MS was performed at the Department of Molecular Biology and Chemistry, The Scripps Research Institute, La Jolla, CA. The instrument was a Hewlett-Packard HP 1100 MSD operated in the negative or positive mode. The HPLC column was removed and the injector output coupled directly to the ESI inlet. Samples (2 μl) were injected in a 90:10 methanol-water (v/v) mobile phase running at a flow rate of 0.35 ml/min. The fragmenter was set at 200 V and the capillary voltage set to 5,000 V. Chromatography was performed before and after solvolysis using dimethoxypropane-HCl (19) to identify sulfates.

To test whether transport of 5α-cyprinol sulfate competed with taurocholate transport by the IPRL, an infusion of 5α-cyprinol sulfate from 20–40 min was superimposed upon a continuous (60 min) infusion of 24-[^{14}C]taurocholate. Output of radioactivity was determined in pools collected every 5 min. Influence of the 5α-cyprinol sulfate infusion on taurocholate uptake was determined by measuring radioactivity in the entering and leaving cannulas.

Transport of 5α-cyprinol by the perfused ileum or jejunum. To test whether 5α-cyprinol was absorbed from the small intestine, it was perfused into the jejunum or the ileum of the anesthetized biliary fistula rat using a recirculating system perfused at 4 ml/min, a rapid perfusion rate that minimizes the unstirred layer effect (27).

The perfusate contained 5α-cyprinol at 0.1 mM concentration and 24-[^{14}C]cholic acid at 0.1 mM, the latter being an absorbable solute that would be quantitatively excreted in bile (in conjugated form). The perfusate contained 130 mM NaCl, 20 mM D-glucose, 1.2 mM Ca^{2+}, and 25 mM tris buffer, as well as phenol red, a nonabsorbable dye, to check paracellular permeability. The jejunal perfusate was adjusted to pH 6.5, the ileal perfusate to pH 7.0. The intestinal segment was perfused for 60 min and bile was collected for 120 min in 10 min pools. A bile sample taken during the steady state of biliary secretion was analyzed for radioactivity as well as by GC-MS to calculate relative rates of absorption.

Effect of 5α-cyprinol sulfate and 5α-cyprinol on taurocholate uptake by COS-7 cells. Competition for taurocholate uptake by 5α-cyprinol sulfate and 5α-cyprinol was examined in COS-7 cells transiently transfected with *asbt*, the conjugated bile acid transporter present in the apical membrane of the ileal enterocyte (28).

Three days later, sodium-dependent taurocholate uptake was determined by incubating transfected COS-7 cells with 1.0 μM [^{3}H]taurocholate in 116 mM NaCl or choline chloride. 5α-Cyprinol sulfate or 5α-cyprinol was added to the incubation buffer at concentrations ranging from 25 μM to 100 μM. After incubating for 15 min at 37°C, the cells were washed three times with 1.0 ml of ice-cold choline containing incubation buffer and lysed with 0.5 ml Triton X-100 in water. Aliquots were taken to determine cell-associated protein and radioactivity.

Fate of 5α-cyprinol sulfate in the carp intestine. Four freshly killed carp were purchased at a Japanese fish market. Gallbladder contents were aspirated for enzymatic determination (23) of the concentration of 5α-cyprinol sulfate. Four other carp were fed and killed 4 h later. The concentration of 5α-cyprinol sulfate in the proximal intestine was determined enzymatically (23). Samples from both proximal and distal intestine were examined by TLC (18) to assess whether 5α-cyprinol was present.

RESULTS

Composition of carp bile

By ESI-MS (negative mode), carp bile contained predominantly (95%) 5α-cyprinol sulfate and 5% of a compound having the molecular weight (515.4) of a C_{27} bile alcohol sulfate with four hydroxy groups. (**Fig. 3**). This compound is most likely 5α-cholestane-3α,7α,12α,26-tetrol (29) esterified with sulfate at C-26 (29). Bile acids were not present, although they have been reported to be present in some samples of carp bile (29). ESI-MS in the positive mode indicated that phospholipids are not present in carp bile. By GC-MS, cholesterol was present in trace amounts.

Physicochemical properties of 5α-cyprinol sulfate and 5α-cyprinol

CMT of 5α-cyprinol sulfate. A solution of 5α-cyprinol sulfate was stable at 4°C, indicating that its CMT was below this temperature.

Ion product of the calcium salt of 5α-cyprinol sulfate. The ion product of $Ca^{2+} \times [5\alpha\text{-cyprinol sulfate}]^{-2}$ was about 9.5×10^{-7} M^3. The calcium salt of 5α-cyprinol sulfate was less soluble than that of glycocholate and taurocholate, but more soluble than that of glycodeoxycholate, whose calcium salt has an ion product of 0.02×10^{-7} M^3 (20).

Solubility of 5α-cyprinol. The aqueous solubility of 5α-cyprinol was 360 μM, a value similar to that of cholic acid (30). Thus 5α-cyprinol was poorly soluble and did not form micelles.

CMC and solubilizing properties of 5α-cyprinol sulfate. **Figure 4** shows the relationship between surface tension and bile salt concentration for the sodium 5α-cyprinol sulfate and sodium taurochenodeoxycholate. The surface tension for each molecule decreased with increasing concentration. The calculated CMC value (see Methods) was about 1.54 mM; this concentration is probably a concentration at which aggregation of monomers begins when Na^+ is present at 0.15 M.

Figure 5 shows the solubilization of Orange OT by the sodium 5α-cyprinol sulfate and sodium taurocholate. The CMC of 5α-cyprinol sulfate by this technique was about 4.5 mM; that of taurocholate was about 9 mM. Both values were obtained with a total [Na^+] of 0.154 M.

Figure 6 shows the solubilization of monooleylglycerol by sodium 5α-cyprinol sulfate and sodium taurocholate. For 5α-cyprinol sulfate, monoolein solubilization began at 1–2 mM, and for taurocholate, at about 3.5 mM. The slope of the solubilization curve (Δ monoolein solubilized/Δ bile salt) was 2.1 for 5α-cyprinol sulfate, a value slightly greater than that observed for taurocholate, which was 1.8.

Metabolism and transport of 5α-cyprinol sulfate and 5α-cyprinol

Metabolism, transport, and choleretic activity of 5α-cyprinol sulfate and 5α-cyprinol in the IPRL. 5α-cyprinol sulfate was infused at a

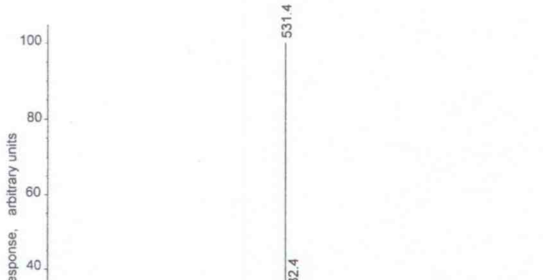

Fig. 3. Electrospray-mass spectrometry (negative mode) of an isopropanol extract of carp bile. The dominant peak has a molecular weight of 531.4 corresponding to that of the anion of 5α-cyprinol sulfate. The minor peak (515.4) is likely to represent the anion of 5α-cholestane-3α,7α,12α-26-tetrol sulfate (29).

Fig. 4. Relationship between surface tension and bile salt concentration for the sodium salt of 5α-cyprinol sulfate and that of taurochenodeoxycholate, a common conjugated C_{24} dihydroxy bile salt. Surface tension was determined by a maximum bubble pressure device (10). The concentration of Na^+ was kept constant at 0.154 M. The critical micellization concentration of both molecules was calculated to be 1.5 mM, a concentration at which monomer aggregation is likely to begin.

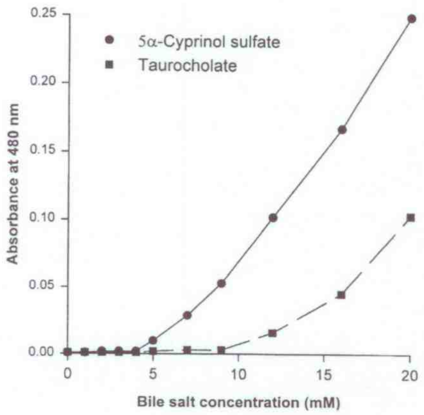

Fig. 5. Relationship between solubilization of Orange OT (1-*O*-tolyl azo-2-naphthol), indicated by absorbance at 483 nm, and bile salt concentration by the sodium salt of 5α-cyprinol sulfate and that of sodium taurocholate. The concentration of Na^+ was kept constant at 0.154 M.

rate of 1 μmol/min/kg for 20 min into the single-pass IPRL. TLC of bile showed two spots. The major spot had the mobility of unchanged 5α-cyprinol sulfate. A minor spot had a slower mobility, compatible with its being a disulfate of 5α-cyprinol sulfate; by its staining properties, it did not contain glucuronic acid. Both spots were eluted, subjected to solvolysis, and rechromatographed. For each spot, the solvolysis product had the mobility of 5α-cyprinol. Thus, 5α-cyprinol sulfate was transported without biotransformation except for a small fraction that underwent additional sulfation.

Recovery of 5α-cyprinol sulfate at the infusion rate of 1 μmol/min/kg was incomplete (65.3 ± 10.2%, n = 6). At the infusion rate of 4 μmol/min/kg, recovery was extremely low (17.0%, mean of two experiments).

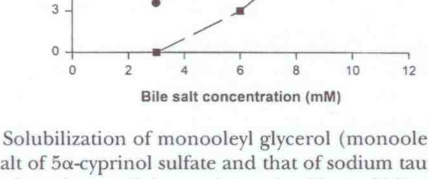

Fig. 6. Solubilization of monooleyl glycerol (monoolein) by the sodium salt of 5α-cyprinol sulfate and that of sodium taurocholate. Solubilization of monoolein was determined by turbidometry.

Because 5α-cyprinol sulfate might undergo hydrolysis during enterohepatic cycling, the metabolism of 5α-cyprinol was also defined in the single-pass IPRL. Bile collected after the infusion of 5α-cyprinol contained no unchanged 5α-cyprinol by TLC (results not shown). HPLC of bile showed the presence of a peak whose retention time was consistent with the taurine conjugate of allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid) and a small unknown peak. Bile was acidified and extracted with diethyl ether in order to identify any unconjugated bile acids or bile alcohols present in bile. This extract was esterified with methanol and the hydroxy groups converted to trimethylsilyl ethers. GC-MS showed a single peak of 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid. The bile acids and alcohols remaining in the aqueous phase after acidification and ether extraction were subjected to a vigorous deconjugation procedure, extracted into ethyl acetate, esterified with methanol, converted to trimethylsilyl ethers, and subjected to GC-MS. Two peaks, in addition to the major endogenous bile acids of the rat (cholic, β-muricholic, and deoxycholic acid) were observed. The major peak was allocholic acid; the minor peak was 3α,7α,12α,26-tetrahydroxy-5α-cholestan-27-oic acid. These data indicate that in the IPRL, when infused at a rate of 1 μmol/min/kg, 5α-cyprinol is converted to two metabolites: allocholic acid (~60%) and the C_{27} bile acid derivative of 5α-cyprinol (~40%). The allocholic acid was efficiently conjugated with taurine. In contrast, the C_{27} bile acid derivative of 5α-cyprinol was amidated with taurine to only a very limited extent and was secreted into bile in mostly unconjugated form. Elution of faint spots on TLC followed by ESI-MS also identified several trace metabolites of 5α-cyprinol. These included an oxo (or unsaturated) derivative, a sulfate conjugate, and a glucuronide conjugate. Because these were such minor pathways, they were not explored further. The metabolism of 5α-cyprinol in the IPRL is summarized in **Fig. 7**.

When 5α-cyprinol sulfate was infused at a rate of 1 μmol/min/kg, hepatic uptake was efficient, being >90% during the 20 min infusion period (results not shown). Biliary secretion of cyprinol sulfate and its disulfate increased, rising to a maximum at 30 min. The maximal rate of secretion was 0.5 μmol/kg/min, and no significant increase in bile flow occurred. When infused at 4 μmol/min/kg, 5α-cyprinol sulfate was again secreted at 0.5 μmol/min/kg, this value appearing to be its T_{max} in the IPRL. Secretion continued at a reduced rate for up to 60 min, indicating that 5α-cyprinol sulfate had been taken up into the hepatocyte and was continuing to be secreted into bile. This dose (4 μmol/min/kg) of 5α-cyprinol sulfate caused bile flow to decrease as soon as it was injected. Bile flow gradually recovered throughout the experiment, even though the secretion rate of 5α-cyprinol sulfate declined.

When 5α-cyprinol, the bile alcohol moiety of 5α-cyprinol sulfate, was infused at a rate of 1 μmol/min/kg, biliary secretion of its two acidic metabolites showed a similar time course, peaking at 30 min. Recovery of 5α-cyprinol in the chemical form of tauroallocholate and the C_{27} cholestanoic acid derivative was complete (101.5% ± 10.6, n = 6). Maximal secretion rate of the two biotransformation products was ~0.9 μmol/min/kg, i.e., similar

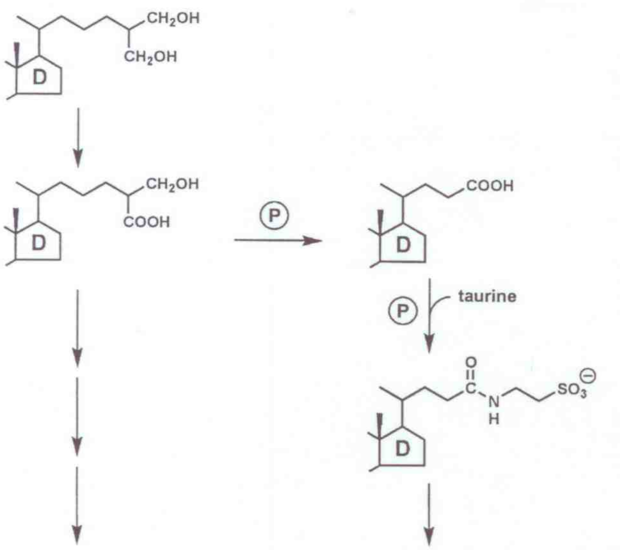

Fig. 7. Biotransformation of 5α-cyprinol in the isolated perfused rat liver when perfused at 1 μmol/min/kg. 5α-Cyprinol is oxidized to its corresponding C_{27} tetrahydroxy cholestanoic acid. The majority of the C_{27} cholestanoic acid enters the peroxisomal compartment (P) where it undergoes oxidative side-chain cleavage to form allocholic acid, which is efficiently amidated with taurine. The remaining C_{27} cholestanoic acid is secreted as such without undergoing conjugation. Two pathways that are not shown and were estimated to be less than 5% of other pathways were conjugation of 5α-cyprinol with glucuronate or sulfate.

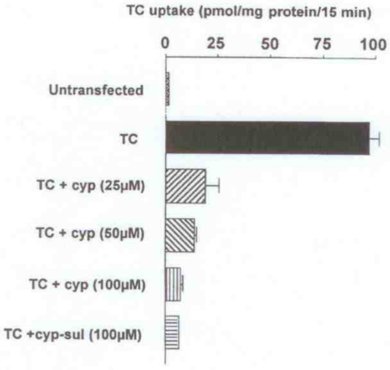

Fig. 8. Inhibition of taurocholate uptake by 5α-cyprinol sulfate and 5α-cyprinol in COS-7 cells transiently transfected with *asbt*, the ileal conjugated bile acid transporter.

to the infusion rate. The infusion of 5α-cyprinol caused a very slight increase in bile flow, consistent with its higher secretion rate (as C_{24} and C_{27} bile acids).

In transport competition experiments using the single-pass IPRL, the imposition of an infusion of 5α-cyprinol sulfate during continuous taurocholate infusion caused a dose-dependent reduction in taurocholate uptake by the liver as well as in taurocholate recovery in bile. The effect on uptake was immediately reversed when the infusion of 5α-cyprinol sulfate was stopped. With the lower dose, there was an immediate, marked increase in taurocholate secretion, indicating storage of taurocholate inside the hepatocyte and subsequent rapid excretion (results not shown).

Intestinal absorption of 5α-cyprinol sulfate and 5α-cyprinol. Because of the poor hepatic secretion of 5α-cyprinol sulfate, its absorption could not be studied by measuring biliary recovery during steady-state ileal perfusion in the anesthetized biliary fistula rat, as is commonly done (27). Interaction of both 5α-cyprinol sulfate and 5α-cyprinol with *asbt* was tested using COS-7 cells transiently transfected with *asbt.* 5α-Cyprinol sulfate at a concentration of 100 μM completely inhibited the uptake of taurocholate at 0.1 μM. Uptake of taurocholate was also inhibited in a concentration-dependent manner by 5α-cyprinol, again with nearly complete inhibition of transport being observed at a concentration of 100 μM. (**Fig. 8**).

Because 5α-cyprinol was rapidly transformed into C_{24} and C_{27} bile acids that were efficiently excreted in bile, it was possible to measure the absorption of 5α-cyprinol from the perfused jejunum and ileum. There was no change in the concentration of phenol red in the perfusate, indicating that paracellular junctions remained intact during the experiment. In the jejunum, the absorption rate of 5α-cyprinol was only 4% that of cholic acid. In the perfused ileum, the absorption rate of 5α-cyprinol was about one-fifth that of cholic acid. The absorption rate of 5α-cyprinol was slightly greater in the ileum than in the jejunum, whereas cholic acid was absorbed twice as rapidly from the jejunum as from the ileum. When infused into the intestine, the biotransformation pattern of 5α-cyprinol was similar to that in the anesthetized biliary fistula animal when 5α-cyprinol was given intravenously. Thus, biotransformation of 5α-cyprinol during transport through the enterocyte is unlikely.

Concentration and biotransformation of 5α-cyprinol sulfate in the carp intestine. The concentration 5α-cyprinol sulfate in the carp gallbladder was 284 ± 48 mM (M ± SD, n = 4). In the intestinal content obtained 4 h after feeding, the concentration of 5α-cyprinol sulfate was 17.4 ± 4.3 mM (M ± SD, n = 5), indicating that mixed micelles are present during digestion. TLC of intestinal contents sampled through the intestine indicated that no 5α-cyprinol was detectable, indicating that bacterial deconjugation of 5α-cyprinol sulfate does not occur during intestinal transit.

DISCUSSION

These experiments indicate that 5α-cyprinol sulfate has excellent physicochemical properties when considered as a digestive surfactant. Its solutions were stable at 4°C, indicating that it is an excellent cold-water detergent. Its calcium salt had a high aqueous solubility, precluding precipitation of its calcium salt in the biliary tract, where the concentration of ionized calcium is about 1 mM (31). As an ester sulfate, its pK_a is <2, and the compound will be fully ionized and soluble at the pH conditions prevailing in the small intestine during digestion (pH5 to pH7).

Despite having the same three nuclear hydroxyl groups as taurocholate, 5α-cyprinol sulfate had a CMC that was considerably lower. The lower CMC of 5α-cyprinol sulfate can be explained by its longer side chain (10). The CMC of the C_{27} (5β) 3α,7α,12α-cholestanoic acid is known to be lower than taurocholate, its C_{24} homolog (32).

5α-Cyprinol sulfate cooperatively associated with unsaturated monoglycerides, forming mixed micelles above 1 mM. It solubilized unsaturated monoglycerides to a greater extent than any of the natural conjugated bile acids present in human bile (21). 5α-Cyprinol sulfate is present in micellar concentrations in the carp intestine and presumably other cyprinid fish, and is thus an efficient agent for promoting the absorption of dietary lipids.

5α-Cyprinol sulfate was not hydrolyzed during hepatocyte transport, but a small fraction was converted to a disulfate. In the IPRL, 5α-cyprinol sulfate was poorly transported into bile as compared with taurocholate. Its T_{max} of 0.5 μmol/min/kg was far below that of taurocholate (7 μmol/min/kg) (22). The prolonged excretion of 5α-cyprinol sulfate after it was removed from the perfusate indicates that in the rat liver, it is poorly transported by *bsep*, the canalicular bile salt pump. Another canalicular transporter, *mrp2*, transports C_{24} amidated conjugated bile acids with a nuclear sulfate group (33), and transport of anions by this canalicular pump also contributes to bile flow. Therefore, inhibition of *mrp*2 by the disulfate metabolite of 5α-cyprinol sulfate could have also contributed to the decrease in bile flow caused by 5α-cyprinol sulfate. Interaction of 5α-cyprinol sulfate with the basolateral uptake transporters mediating taurocholate import into the hepatocyte (*ntcp* and possibly one or more *oatp*s) was also shown by the IPRL studies. Presumably, 5α-cyprinol sulfate is well transported by the carp hepatocyte.

In contrast to the minimal hepatic biotransformation of 5α-cyprinol sulfate, 5α-cyprinol underwent oxidation of its C-27 primary alcoholic group to a carboxyl group, thereby forming a C_{27} tetrahydroxy 5α-cholestanoic acid. This oxidation can be mediated by cholesterol 27-hydroxylase, a mitochondrial enzyme (34, 35), but other nonmitochondrial pathways for formation are possible. 5α-Cyprinol also underwent side-chain cleavage to form allocholic acid, which was then conjugated with taurine. These steps take place in peroxisomes (36). 5β-Cyprinol, the A/B isomer of 5α-cyprinol, was reported many years ago to be converted in rats (37) and guinea pigs (38) to cholic acid. Taken together, these results indicate that in the rat, the C_8 side chain of 5α-cyprinol is less readily oxidatively cleaved to form an isopentanoic acid side chain than that of its 5β (A/B *cis*) epimer. The C_{27} 5α-cholestanoic acid was secreted into bile in unconjugated form, presumably because the presence of a hydroxy group at C-26 (a β-hydroxy group) renders the molecule a poor substrate for the recently identified C_{27} CoA ligase (39). The addition of a β-hydroxy group to chenodeoxycholic acid results in incomplete conjugation with taurine during hepatic transport (40). Efficient transport of tauroallocholate in the anesthetized biliary fistula rat has recently been reported (41) in agreement with our results using the perfused liver.

Both 5α-cyprinol sulfate and 5α-cyprinol interacted with *asbt*. Taurocholate transport was greatly reduced by both 5α-cyprinol sulfate and 5α-cyprinol. These findings suggest that in humans, ingested 5α-cyprinol sulfate should be absorbed via *asbt*.

An unexpected finding was the extremely low intestinal absorption rate of 5α-cyprinol as compared with that of cholic acid. Passive transcellular absorption of uncharged molecules is considered to increase with molecular volume and decrease with the polar surface area (42, 43). For both of these parameters, there should be little difference between 5α-cyprinol and protonated cholic acid. Therefore, the much greater rate of absorption of cholic acid than of 5α-cyprinol, especially in the jejunum, strongly suggests the presence of one or more intestinal transport systems for cholic acid in the rat small intestine. There is indirect evidence for cholic acid absorption being carrier mediated in the hamster small intestine (44).

While this work was in progress, 5β-scymnol sulfate, a bile alcohol sulfate that is present in cartilaginous fish and that has a chemical structure similar to 5β-cyprinol sulfate, was shown to undergo an enterohepatic circulation in *Raja erinacea*, the little skate. 5β-Scymnol sulfate undergoes vectorial hepatocyte transport by basolateral and apical transporters related to those that mediate the hepatocyte transport of C_{24} bile acids in mammals (45).

5α-Cyprinol sulfate, which we found to be present in gallbladder bile at the high concentration of 0.3 M, has been shown to be the toxic constituent of carp bile (16). We found that the intravenous infusion of 5α-cyprinol sulfate at a physiological rate caused hemolysis and death in the rat. 5α-Cyprinol sulfate is more surface active than taurocholate (46) and is likely to be more hemolytic. The oral toxicity of 5α-cyprinol sulfate in humans (17) probably results from its being transported by the ileal transport system for conjugated bile acids at a rate exceeding its T_{max} for canalicular secretion. As a result, it accumulates in the hepatocyte and plasma, causing hepatotoxicity and hemolysis. A similar imbalance between ileal absorption and hepatic secretion can be observed in the rat when taurine dihydroxy bile acids are infused into the ileum. Absorption occurs at a rate exceeding that of hepatic clearance, and frank toxicity is observed (27). In humans, renal toxicity is a hallmark of 5α-cyprinol sulfate ingestion (17). If 5α-cyprinol sulfate were to enter the glomerular filtrate, it could be transported into the renal tubular cells by *asbt*, which is present on the apical membrane (47). The lack of toxicity of 5α-cyprinol when compared with its sulfate (14) can now be explained by the poor absorption of the free alcohol as well as its efficient hepatic biotransformation to bile acids that are readily secreted into bile.

Lastly, our work shows a major difference in metabolism between bile alcohol sulfates and C_{24} conjugated bile acids. 5α-Cyprinol sulfate does not undergo bacterial hydrolysis during intestinal passage, at least in the Asiatic carp. If it were to undergo bacterial hydrolysis, as is likely to occur in herbivorous cyprinids, 5α-cyprinol would be formed. Our data indicate that 5α-cyprinol has a low aqueous solubility, is nonfunctional, and is rapidly lost from the organism. In contrast, when C_{24} conjugated bile acids undergo bacterial deconjugation in the distal small intestine, the unconjugated moiety that is formed is soluble at the alkaline pH of the ileum. Unconjugated bile acids are absorbed by passive as well as carrier-mediated mechanisms and are reconjugated in the liver, thereby protecting the integrity of the circulating bile acid pool (48, 49). Thus, for maintaining the bile acid pool in the presence of deconjugating bacteria in the small intestine, bile acid amidates appear to be better than bile alcohol sulfates. This functional superiority of C_{24} conjugated bile acids may explain their evolution from bile acids in animals possessing a deconjugating flora in the distal intestine.

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