3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-sulfonate: synthesis and suitability for the study of cholate transport[1]

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Abstract In order to facilitate the study of transport processes of unconjugated C-24 bile salts, simple syntheses of 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-sulfonate (norcholansulfonate) and 3α,7α,12α-trihydroxy-24-nor-5β-[7β-^{3}H]cholan-23-sulfonate were devised. The hydrophilic–hydrophobic properties of norcholansulfonate, as determined by its chromatographic behavior as well as by its partition between 1-octanol and water, are more similar to those of cholyltaurine than to those of cholate. Self-association of norcholansulfonate in phosphate buffer, pH 7.4, with an ionic strength of 150 mM begins at a concentration of about 1 mM, comparable to that of cholyltaurine and cholate, as determined by spectral changes in fluorescence emissions of {N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7b-amino-3a,12a-dihydroxy-5b-cholan- 24 -oyl}-2′-aminoethanesulfonate (7β-NBD-NCT). The apparent CMC value obtained from solubilization of the dye Orange OT, 8.5 mM, is comparable to that of cholyltaurine, 7.5 mM, and lower than that of cholate, 9.5 mM. Norcholansulfonate is readily taken up by rat liver and completely excreted unmetabolized into bile with about the same secretion maximum (T_m) as cholyltaurine. Biliary excretion of norcholansulfonate is inhibited by cholyltaurine, and, vice versa, norcholansulfonate inhibits cholyltaurine secretion. Concerning metabolism and excretion, norcholansulfonate with the sulfonate group in the position where cholate has the carboxylate group should behave as an appropriate cholate analogue in mediated transport processes.—**Schwab, D., H. Thom, J. Heinze, and G. Kurz.** 3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-sulfonate: synthesis and suitability for the study of cholate transport. *J. Lipid Res.* 1996. **37:** 1045–1056.

Supplementary key words hepatobiliary transport • bile salt secretion • fluorescent bile salt derivative • critical micellar concentration • self-association

Conjugated bile salts are, in the course of their enterohepatic circulation, partially subject to deconjugation by enteral microorganisms. Consequently, not only conjugated but also unconjugated bile salts reach the liver and are taken up into hepatocytes. How unconjugated bile salts are taken up into hepatocytes has not been definitively elucidated with regard to the extent of membrane diffusion and the number and specificity of transport systems involved. Taurine-conjugated bile salts, the corresponding acids of which have pK_a values < 2 (1, 2), cross the plasma membrane of hepatocytes by mediated transport (3). In contrast, unconjugated bile salts, the corresponding acids of which exhibit as carbonic acid pK_a values about 5 (2, 4, 5), are also able to freely diffuse across biological membranes. Such a diffusional component makes the investigation of hepatocellular transport processes more complicated, especially if more than one transport system is involved, as for taurine-conjugated bile salts (3).

In order to compare the uptake of unconjugated and taurine-conjugated bile salts and to scrutinize whether they share the same sinusoidal transport systems, we synthesized norcholansulfonate for kinetic transport studies. Norcholansulfonate has a sulfonate group in the position where cholate bears a carboxylate group and, due to the fact that the structure of the steroid nucleus, the length of the side chain, and the distance of the negative charge from the nucleus are the same as in

Abbreviations: CMC, critical micellar concentration; DCI, direct chemical ionization; EI, electron impact ionization; ESI, electrospray ionization; HPLC, high pressure liquid chromatography; HPTLC, high performance thin-layer chromatography; NBD, 7-(4-nitrobenzo-2-oxa-1,3-diazol); 7β-NBD-NCT, {N-[7-(4-nitrobenzo- 2-oxa-1,3-diazol)]-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oyl}-2′-aminoethanesulfonate; norcholansulfonate, 3α,7α,12α-trihydroxy -24-nor-5β-cholan-23-sulfonate; [7β-^{3}H]norcholansulfonate, 3α,7α, 12α-trihydroxy-24-nor-5β-[7β-^{3}H]cholan-23-sulfonate; TLC, thin-layer chromatography.

[1]Dedicated to Professor Dr. Wolfgang Gerok on the occasion of his 70th birthday.

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cholate, it is referred to as an isogeometric derivative of cholate, even with the different geometric structures of the anionic groups (**Fig. 1**). Alkane sulfonic acids are strong acids (6–8), and consequently norcholansulfonate exists as the salt of a sulfonic acid under physiological conditions, almost exclusively in the deprotonated anionic form. Accordingly, norcholansulfonate, which is unconjugated, has properties similar to the conjugated bile salt cholyltaurine, both compounds being sulfonates, and neither compound is capable of undergoing transport by simple diffusion through membranes. A comparative study of the uptake of norcholansulfonate and cholate, both structural unconjugates, and taurine-conjugated bile salts should show the influence on hepatic uptake of side chain length and the difference between a sulfonate and carboxylate group. The present communication describes a simple synthesis of norcholansulfonate and characterizes those properties necessary for the realization of transport studies. In a preliminary communication, sinusoidal transport of norcholansulfonate was compared with that of cholate (9).

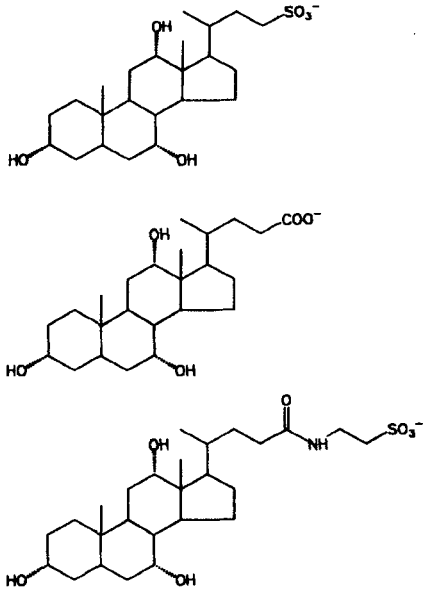

Fig. 1. Comparison of the chemical structure of norcholansulfonate (top) with that of cholate (middle) and of cholyltaurine (bottom).

MATERIALS AND METHODS

Animals

Male Wistar S 300 rats (Interfauna, Tuttlingen, Germany) weighing 200–250 g were used. The animals had free access to food (standard rat diet Altromin 300 R, Altromin GmbH, Lage, Germany) and tap water, and were housed in a constant temperature environment with natural day–night rhythm.

Materials

Cholate (Na^+-salt), cholyltaurine (Na^+-salt), and Orange OT were purchased from Sigma Chemie (Taufkirchen, Germany). Orange OT was crystallized from acetone–water to obtain a melting point of 131°C. Collagenase "Worthington" CLS II with a specific activity of 150–200 U/mg protein was obtained from Biochrom (Berlin, Germany). Silicone oils AR 20 and AR 200 were obtained from Wacker Chemie (München, Germany). Trypan blue, and Amberlite® XAD-2 were from Serva (Heidelberg, Germany). Cholic acid, silica gel 60 (40–63 μm and 63–200 μm), silica gel plates for TLC (Kieselgel 60, 20 × 20 cm), and HPTLC (Kieselgel 60, 10 × 20 cm) were purchased from Merck (Darmstadt, Germany). [G-^{3}H]cholyltaurine (74 GBq/mmol) and sodium boro[^{3}H]hydride (496 GBq/mmol) were obtained from Amersham Buchler (Braunschweig, Germany). 7β-NBD-NCT was synthesized as described (10).

All other chemicals were of the highest quality available from commercial sources.

Molecular orbital calculations

Semiquantitative molecular orbital calculations were carried out by the AM1 method using the program MOPAC V. 2.10., QCPE 464 (Quantum Chemistry Program Exchange, Indiana University, Indianapolis, IN).

Protein determination and detection of radioactivity

Protein was determined by means of a modified biuret method (11) using chloroform instead of ether to remove turbidity due to lipid. Bovine serum albumin was used as the standard.

Radioactivity of liquid samples was determined by liquid scintillation counting (Liquid Scintillation Counter Wallac 1411, Berthold, Wildbad, Germany), using quench correction and external standard for absolute radioactivity determination. Four ml of Aquasafe 300 (Zinsser Analytic, Frankfurt, Germany) was added routinely to aqueous and methanolic solutions containing tritiated compounds; for determination of 1-octanol/water partition coefficients, Ultima Gold™ (Packard, Groningen, Netherlands) was used as scintillator. Radioactivity on TLC plates was detected using a radiochromatogram scanner (Linear Analyzer LB 284, Berthold), equipped with a proportional counter LB 2821 HR (Berthold).

Determination of 1-octanol/water partition coefficient

The 1-octanol/water partition coefficients were determined using 37 kBq of the corresponding tritium-labeled bile salts (12). The bile salts were dissolved in 600 μl of 100 mM sodium-phosphate buffer, pH 7.4, presaturated with 1-octanol, and 600 μl of 1-octanol, presatu-

rated with water, was added. The samples were left to equilibrate for 4 days under vigorous shaking at 25°C. After centrifugation the two phases were carefully separated and the radioactivity was determined.

Reversed phase HPLC

Isolation of radioactive labeled and detection of non-labeled bile salt derivatives by C-18 reversed phase HPLC were carried out isothermally at 35°C using a liquid chromatograph LKB 2150 (Pharmacia LKB, Freiburg, Germany) equipped with a Rheodyne injector (Cotati, CA) and a 4.6 × 250 mm-symmetry™column, particle size 5 μm (Millipore, Eschborn, Germany), with a flow rate of 1 ml/min. Unlabeled bile salts were monitored at 210 nm with a 2140 rapid spectral detector (Pharmacia LKB, Freiburg, Germany), and radioactive labeled bile salts were detected with a radio-flow-through-monitor (Ramona-90, Raytest, Straubenhardt, Germany). Analytical determinations were carried out by continuous liquid scintillation counting with a flow rate of the liquid scintillator (Quickszint Flow 302, Zinsser Analytic, Frankfurt, Germany) of 2.5 ml/min and preparative separations were followed by a solid phase glass scintillator (Raytest). The mobile phase consisted of methanol–0.01 M potassium phosphate, pH 7.4, 65:35 (v/v) for analytical procedures, and of methanol–water–trifluoracetic acid 60:40:0.1 (v/v/v) for preparative procedures.

Determination of self-association

Self-association of bile salts was followed by fluorescence studies (13). The fluorescence studies were carried out with the fluorescent bile salt derivative 7β-NBD-NCT, because the fluorescence of the 4-nitrobenzo-2-oxa-1,3-diazol fluorophore depends on the hydrophobicity of the environment (10, 14–16). Fluorescence spectra of 5–10 μM 7β-NBD-NCT solutions in the presence of different bile salt concentrations were determined at 25°C in 3-ml quarz cuvettes with a spectrofluorimeter (MPF 44 A, Perkin-Elmer, Stuttgart, Germany), connected with a DCSU-2 differential spectra unit and corrected with Basic Blue 3 (2,7-bis-(diethylamino)-phenazoxonium-chloride) (17). The excitation wavelength was 495 nm and the fluorescence spectra were recorded from 500 to 740 nm. Fluorescence quantum yields were determined by using the quantum counter 9,10-diphenylanthracene in cyclohexane (18). Ultraviolet absorption spectra were measured with a UV/Vis spectrometer (Lambda 5, Perkin-Elmer, Stuttgart, Germany). Quantum yields were calculated taking equation 1 as the basis:

$$\phi_p = \phi_{st} \frac{n^2{}_p \cdot A_p \cdot \left(1 - 10^{-E_{st}}\right)}{n^2{}_{st} \cdot A_{st} \cdot \left(1 - 10^{-E_p}\right)} \qquad Eq1)$$

where p, probe; st, standard; ϕ, quantum yield; n, refraction index at 20°C and 589 nm; A, area of the emission spectrum; and E, extinction at 495 nm.

Determination of apparent CMC

The apparent CMC of bile salts was determined in phosphate buffer, pH 7.4, adjusted with NaCl to the ionic strength of 150 mM, by means of dye solubilization using Orange OT as indicator dye, exactly as described (19).

Isolation of hepatocytes

Isolation and characterization of hepatocytes from rat liver were carried out as recently described (3).

Liver infusion experiments and incubation of hepatocytes

Constant infusion of cholyltaurine and norcholansulfonate with tracer doses of the respective radioactive labeled analogue, single bolus experiments, and estimation of biliary elimination maxima (T_m) were performed as described (14, 20). Bile was collected at different times beginning 10 min before the start of an injection. The nature of the excreted compounds was determined by HPTLC, analyzing the methanolic extracts of lyophilized bile fractions collected in 5-min intervals.

Metabolites of cholate and norcholansulfonate in isolated hepatocytes were identified after incubating the hepatocytes for different lengths of time in suspensions of 1×10^6 to 5×10^6 cells/ml with 5 or 100 μM of the respective bile salt containing 370 kBq of its tritium-labeled derivative. Separation of hepatocytes from the extracellular medium was performed by centrifugation through the layer of silicone oil into a buffered solution of 4% NaCl as lower layer. Immediately after centrifugation the lower part of the centrifugation tube was shock-frozen in liquid nitrogen, the supernatant medium was collected, and the lower part of the tube containing the cell pellet was cut off. After lyophilization of the probes to be analyzed, bile salts were extracted with methanol. For HPTLC analysis, extracts from cell pellets were pre-purified by C-18 reversed phase extraction (Worldwide Monitoring™, United Chemical Technologies, Bristol, PA), using methanol as elution solvent.

Analysis of organic compounds

Elemental analyses were carried out with a Perkin-Elmer 240 analyzer (Perkin-Elmer, Überlingen, Germany). ^{1}H-NMR-spectra were measured on a 250-MHz-NMR

spectrometer (Spektrospin WM 250, Brucker, Karlsruhe, Germany). Two-dimensional ^{1}H–^{13}C-correlated-NMR-spectra were recorded on a 400-MHz-NMR spectrometer (Brucker) (21). Values were in parts per million relative to tetramethylsilane as internal standard. Mass spectra were recorded with a Finnigan MAT 44S mass spectrometer connected with a data unit SS 2000 (Finnigan, San Jose, CA). Ionization was performed either by EI (electron impact ionization) with an electron energy of 70 eV or by DCI (direct chemical ionization) with an electron energy of 70 eV, using ammonia as reactant gas at a pressure of 30 Pa. In both cases positive ions were recorded (22–24). Mass spectra with electrospray ionization (ESI) of bile salt sulfonates were recorded on a Finnigan TSP 7000 triple quadrupole mass spectrometer (Finnigan) operating in the negative ion mode (25). The instrument was controlled and data were analyzed using ICIS software (Finnigan). The electrostatic-spray ion source was operated at -4.5 kV, and the atmosphere-vacuum transfer capillary was heated at 220°C. Full scan mass spectra were recorded from mass-to-charge ratio (*m/z*) 300 to 1100 in 2 sec. Samples were dissolved in acetonitrile–water 7:3 (v/v) and infused at a flow rate of 25 µl/min. Bile acids and their derivatives were detected on TLC plates by spraying the dried plates with concentrated sulfuric acid and subsequently heating them at 120°C for 5 min.

Syntheses

Column chromatography under hydrostatic pressure was performed on 100 × 5 cm columns of silica gel 60 (63–200 µm), and flash chromatography (26) on 10 × 5 cm columns (40–63 µm). Solvent systems for chromatographic separations were: solvent system 1, ethyl acetate–cyclohexane–acetic acid 100:40:1 (v/v/v); solvent system 2, cyclohexane–ethyl acetate 15:2 (v/v); solvent system 3, ethyl acetate–cyclohexane–acetic acid 23:7:3 (v/v/v); solvent system 4, chloroform–methanol–acetic acid 7:2:0.5 (v/v/v); solvent system 5, 1-butanol–acetic acid–water 9:2:1 (v/v/v).

3α,7α,12α-Triformoxy-5β-cholan-24-oic acid (I) (**Fig. 2**). The synthesis of triformylated cholic acid was carried out exactly as described (27, 28). All analytical data were consistent with the reported values.

3α,7α,12α-Triformoxy-24-nor-5β-cholan-23-chloride (II) (Fig. 2). The 23-chloride of 3α,7α,12α-triformoxy-24-nor-5β-cholan (II) was synthesized as described (29), starting with 3α,7α,12α-triformoxy-5β-cholan-24-oic acid (I) (20 g, 40.6 mmol). The crude product was purified by flash chromatography using solvent system 2 and yielded 15 g (31.2 mmol, 77% yield) of the pure product. MP, 167°C; TLC: R_f = 0.72 (solvent system 1), 0.09 (solvent system 2), 0.85 (solvent system 3); ^{1}H-NMR ($CDCl_3$): δ = 0.80 (s, CH_3–18), 0.88 (d, J = 5Hz, CH_3–21), 0.96 (s, CH_3–19), 3.49/3.61 (m, b, CH_2–23), 4.73 (m, b, CH–3), 5.09 (s, CH–7), 5.29 (s, CH–12), 8.03 (s, OCHO at C–3), 8.10 (s, OCHO at C–7), 8.17 (s, OCHO at C–12); mass spectrum (EI): *m/z* = 390 M-(2 HCOOH), 344 M-(3 HCOOH), 299 M-(2 HCOOH + side chain), 253 M-(3 HCOOH + side chain); mass spectrum (DCI): *m/z* = 500 $(M+NH_4)^+$; anal. calcd. for $C_{26}H_{39}O_6Cl$ (483.0): C, 64.6, H, 8.1; found: C, 64.45, H, 8.05.

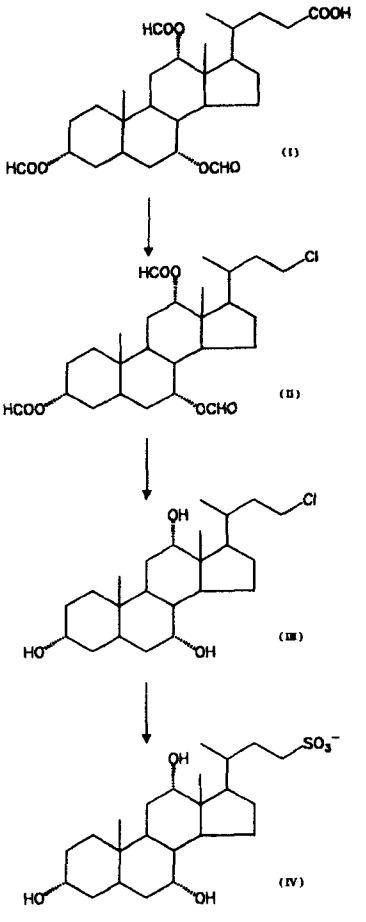

Fig. 2. Synthesis of norcholansulfonate (3α,7α,12a-trihydroxy-24-nor-5β-cholan-23-sulfonate); (I), 3α,7α,12α-triformoxy-5β-cholan-24-oic acid; (II), 3α,7α,12α-triformoxy-24-nor-5β-cholan-23-chloride; (III), 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-chloride; (IV), norcholansulfonate.

3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-chloride (III) (Fig. 2).3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-chloride (II) (10g, 20.7 mmol) was dissolved in 50 ml of a solution of 10% NaOH in methanol (w/v), and the mixture was stirred for 2 h. The resulting suspension was evaporated under reduced pressure to dryness, the residue was dissolved in diethylether, and the solution was washed three times with water to neutrality. The combined organic phases were dried over Na_2SO_4, fil-

tered, and evaporated under reduced pressure to dryness. The crude product was purified by flash chromatography using solvent system 2. The yield of the pure product was 7.4 g (18.6 mmol, 90% yield). MP, 182°C; TLC: R_f = 0.04 (solvent system 1), R_f = 0.28 (solvent system 3); ^{1}H-NMR ($CDCl_3$): δ = 0.73 (s, C$\underline{H}_3$–18), 0.90 (s, C$\underline{H}_3$–19), 1.04 (d, J = 5Hz, C$\underline{H}_3$–21), 3.84 (s, C$\underline{H}$–7), 3.99 (s, C$\underline{H}$–12); mass spectrum (EI): *m/z* = 362 M-(2 H_2O), 344 M-(3 H_2O), 271 M-(2 H_2O + side chain), 253 M-(3 H_2O + side chain); mass spectrum (DCI): *m/z* = 416 $(M+NH_4)^+$, 363 $(M+NH_4-H_2O-Cl)^+$, 345 $(M+NH_4-2H_2O-Cl)^+$; anal. calcd. for $C_{26}H_{39}O_6Cl$ (399.0): C, 69.2, H, 9.8; found: C, 69.0, H, 9.9.

Sodium 3α,7α,12α-trihydroxy-24-nor-5β -cholan-23-sulfonate (IV) (Fig. 2). 3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-chloride (III) (4 g, 10.0 mmol) was dissolved in 200 ml ethanol, and 40 ml of a 5% aqueous solution of Na_2SO_3 was added. The mixture was heated at reflux temperature for 8 h. The solution was filtered and the filtrate was evaporated under reduced pressure. The crude product was purified by flash chromatography using solvent system 4 and yielded 3.6 g (7.5 mmol, 75% yield) of the pure product. The product obtained after chromatography was finally freed from silica gel with Amberlite® XAD-2 or by C-18 reversed phase chromatography. MP, > 280°C; TLC: R_f = 0.10 (solvent system 4), 0.30 (solvent system 5); ^{1}H-NMR (CD_3OD): δ = 0.73 (s, C$\underline{H}_3$–18), 0.92 (s, C$\underline{H}_3$–19), 1.04 (d, J = 7Hz, C$\underline{H}_3$–21), 2.73/2.91 (m, b, C$\underline{H}_2$–23), 3.36 (m, b, C$\underline{H}$–3), 3.79 (s, C$\underline{H}$–7), 3.95 (s, C$\underline{H}$–12); mass spectrum (ESI): *m/z* = 443.1, expected 443.2.

Sodium 3α,12α-dihydroxy-7-oxo-24-nor-5β-cholan-23-sulfonate. Sodium 3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-sulfonate (IV) (500 mg, 1.0 mmol) was dissolved in 10 ml of 40% aqueous acetone, and N-bromosuccinimide (300 mg, 1.6 mmol) was added. The orange-colored mixture was stirred until decoloration, at least 30 min. After evaporation of the solvent under reduced pressure, the crude product was purified by flash chromatography using solvent system 5. The yield of the pure product was 300 mg (0.62 mmol, 60% yield). MP, 258°C; TLC: R_f = 0.22 (solvent system 4), 0.36 (solvent system 5); ^{1}H-NMR (CD_3OD): δ = 0.74 (s, C$\underline{H}_3$–18), 1.06 (d, J = 7Hz, C$\underline{H}_3$–21), 1.22 (s, C$\underline{H}_3$–19), 2.56 (t, J = 11Hz, C$\underline{H}$–8), 2.71/2.90 (m, b, C$\underline{H}_2$–23), 2.98 (dd, 7/14Hz, C$\underline{H}$–6_{ax}) 3.54 (m, b, C$\underline{H}$–3), 4.00 (s, C$\underline{H}$–12); mass spectrum (ESI): *m/z* = 441.1, expected 441.2.

3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid was synthesized exactly as described (30). All analytical data were consistent with the reported values. The ^{1}H-NMR-spectrum could be further resolved by two-dimensional ^{1}H–^{13}C-correlated-NMR-spectra: ^{1}H-NMR ($CDCl_3$): δ = 0.72 (s, C$\underline{H}_3$–18), 1.02 (d, J = 7Hz, C$\underline{H}_3$–21), 1.24 (s, C$\underline{H}_3$–19), 2.56 (t, C$\underline{H}$–8), 2.98 (dd, J = 7/14 Hz, C$\underline{H}$–6_{ax}), 3.52 (m, b, C$\underline{H}$–3), 4.00 (s, C$\underline{H}$–12).

Sodium 3α,7α,12α-trihydroxy-24-nor-5β-[7β-^{3}H]cholan-23-sulfonate. Sodium 3α,12α-dihydroxy-7-oxo-24-nor-5β-cholan-23-sulfonate (17,0 mg, 214 μmol) was dissolved in 0.5 ml of 5% aqueous $NaHCO_3$. The solution was added to a freshly opened ampoule of sodium boro[^{3}H]hydride (3.7 GBq, 496 GBq/mmol), and the reaction mixture was allowed to stand at room temperature for 8–12 h. In order to remove sodium boro[^{3}H]hydride that may have not reacted, 25 μl of an aqueous solution of 1 M D-glucose, pH 7.5, was added. After 2 h the solvent was evaporated during centrifugation under reduced pressure, and the tritium-labeled compound was subsequently purified either by HPTLC, using solvent system 5, or HPLC, using methanol–water–trifluoracetic acid 60:40:0.1 (v/v/v). The radiochemical yield of the stereochemical pure product was 0.37 GBq (124 GBq/mmol, 10% yield).

Sodium 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholan-24-carboxylate. 3α,12α-Dihydroxy-7-oxo-5β-cholic acid (12 mg, 25 μmol) was dissolved in a mixture of 250 μl of THF and 25 μl of 1 N NaOH. The solution was added to a freshly opened ampoule of sodium boro[^{3}H]hydride (3.7 GBq, 370 GBq/mmol), and the reaction mixture was allowed to stand at room temperature for 8–12 h. In order to remove sodium boro[^{3}H]hydride that might have not reacted, 25 μl of an aqueous solution of 1 M D-glucose, pH 7.5, was added. After 2 h the solvent was evaporated during centrifugation under reduced pressure, and the tritium-labeled compound was subsequently purified either by HPTLC, using solvent system 3, or HPLC, using methanol–water–trifluoracetic acid 75:25:0.1 (v/v/v). The radiochemical yield of the stereochemical pure product was 1.1 GBq (92.5 GBq/mmol, 30% yield).

Purification of bile salts

Norcholansulfonate, 7β-NBD-NCT, as well as the commercially available bile salts cholate and cholyltaurine were purified by C-18 reversed phase chromatography (Worldwide Monitoring™, United Chemical Technologies, Bristol, PA), using approximately 70 mg of the respective bile salt in water and a gradient of water–methanol from 100:0 to 50:50 (v/v) for elution. 7β-NBD-NCT was alternatively purified by HPLC using methanol–water 60:40 (v/v) as mobile phase.

Purity was confirmed by TLC and HPTLC analysis, which resulted with all bile salts in only one spot when 100 μg of the respective bile salt was chromatographed. Purity of radioactive labeled bile salts was verified by HPLC.

RESULTS

Structural comparison of the sulfonate and the carboxylate groups

The electronic properties of the sulfonate and those of the carboxylate group were compared by semiquantitative molecular orbital calculations, which were performed because of their simplicity on the basis of the corresponding methyl derivatives (**Table 1**). As expected, the two groups bearing the same total charge differ in their geometry, being planar for the carboxylate and tetrahedral for the sulfonate. Despite the clear differences in binding lengths and partial charges, and despite the higher space requirement of the sulfonate group, the space filling models computed on the basis of the semiquantitative molecular orbital calculations (**Fig. 3**) illustrate that these differences are not too large for the assumption that norcholansulfonate may act as a competing substrate for cholate. Above all, the relative positions of the center of the negative charges are comparable, so that the term isogeometric compounds is fully justified.

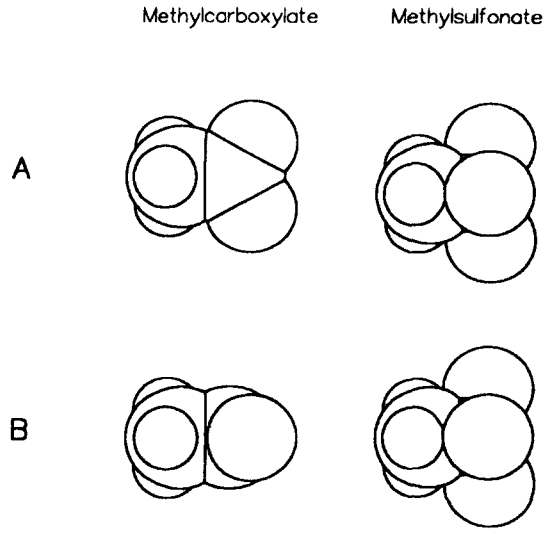

Fig. 3 Comparison of the methyl deriviatives of carboxylate and sulfonate, Structures were computed from semiquantitative molecular orbital caluculations on the basis of the methyl derivates. A: The planar carboxylate group is placed in the plane of drawing. B: The planar carboxylate group is situtaed perpendicular to the plane of drawing.

Synthesis of norcholansulfonate

Norcholansulfonate was synthesized in a simple 4-step procedure (Fig. 2), starting with cholic acid as a reasonably accessible starting material. The crucial step in synthesis, the shortening of the side chain by one C-atom and the transformation of C-23 to a reactive intermediate, allowing the formation of the corresponding sulfonate, was performed using the Kochi reaction (31) and yielded 77% of the 3α,7α,12α-triformoxy-24-nor-5β-cholan-23-chloride (Fig. 2, II). Removal of the protecting formyl groups and nucleophilic substitution by sulfite occurred without any difficulties. The introduction of tritium in the 7β-position turned out to be easily executed by reducing the 7-oxo derivatives with sodium boro[^{3}H]hydride (27), and resulted in the formation of 10% of [7β-^{3}H]norcholansulfonate and 30% of [7β-^{3}H]cholate, respectively.

TABLE 1. Numeric values of the semiquantitative molecular orbital calculations of the methyl derivates of the carboxylate and sulfonate qroup

		$CH_3-CO_2^-$		$CH3-SO_3^-$
Geometry of the anion		planar		tetrahedron
Binding length	*C–O*	127 pm	*S–O*	140 pm
	C–COO	153 pm	*C–SO*$_3$	176 pm
Binding angle	OCO	118°	OSO	105°
Total charge		-1		-1
Partial charge	*C*	+0.32	*S*	+2.86
	$O_{1,2}$	-0.60	$O_{1,2,3}$	-1.08
Enthalpy of formation		-483 KJ		-694 KJ
Electron energy		-2363 eV		-3870 eV
Ionization potential		-3,96 eV		-6.41 eV

Calculations were carried out by the AM1 method using the program MOPAC V. 2.10. Differences of ≤ 0.1° in angles were omitted.

Evaluation of norcholansulfonate hydrophobicity

As reversed phase HPLC mobility gives a rough indication of the behavior of a compound in membrane permeation by simple diffusion, norcholansulfonate and cholate were compared with cholyltaurine on a C-18 reversed phase column, using methanol–0.01 M potassium phosphate, pH 7.4, 65:35 (v/v) as mobile phase. Whereas the retention time of 12.0 min for norcholansulfonate was only insignificantly smaller than that determined for cholyltaurine, being 12.3 min under the experimental conditions used, the retention time of 17.0 min for cholate was clearly higher (**Table 2**). The comparison of the relative mobility on HPTLC indicates likewise that norcholansulfonate and cholyltaurine are similar and differ considerably from cholate (Table 2). In order to confirm that norcholansulfonate is more similar to cholyltaurine than to cholate with regard to its hydrophobicity, the 1-octanol/water partition coefficients were determined and compared. The determinations were carried out by using 100 mM sodium phosphate buffer, pH 7.4, as aqueous phase, and the bile salts were applied in concentrations far below the apparent CMC of the corresponding anions, using solely the tritium-labeled derivatives at initial concentrations of about 1 μM. The partition coefficients of norcholansulfonate and cholyltaurine, being 0.31 and 0.30, respectively, differ significantly from that of cholate, being 4.75 (Table 2). Whereas the partition coefficient of cholyltaurine is in excellent agreement with the reported value, the partition coefficient for cholate differs by a factor of 2.6 (12).

Evaluation of self-association of norcholansulfonate

With increasing concentrations in aqueous solutions, bile salts exhibit the tendency to self-association (2, 13, 19, 32–34). In order to evaluate the range useful for kinetic transport studies in which norcholansulfonate exists as a true monomer, self-association of norcholansulfonate was determined by means of dye solubilization using Orange OT (19) and by fluorescence enhancement (13) using the fluorescent bile salt derivative 7β-NBD-NCT (10). The amount of Orange OT solubilized in dependency on bile salt concentration was determined photometrically by plotting the extinctions of Orange OT versus bile salt concentration (**Fig. 4**). The intersection point of the line parallel to the abscissa with the line drawn through the first 3–5 points when the solubilization of the dye begins to increase was taken as apparent CMC. The apparent CMC of norcholansulfonate (Table 2) lies between the values of cholyltaurine and cholate.

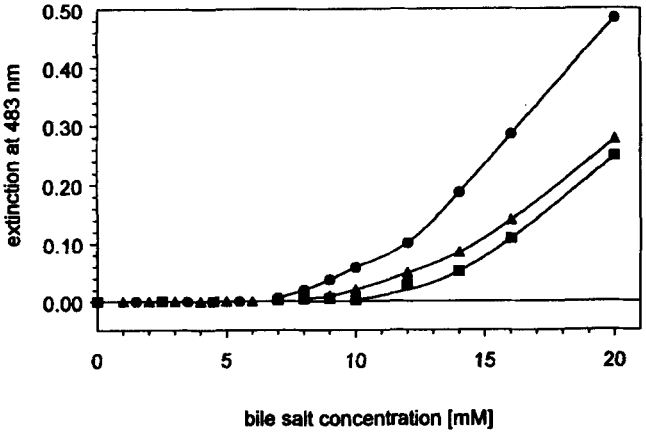

Fig. 4. Effect of norcholansulfonate (▲), cholyltaurine (●), and cholate (■) concentration on solubilization of Orange OT. Adsorbance of Orange OT was measured at 483 nm (see Materials and Methods for details). For the sake of clarity, in the concentration range from 0–7 mM, not all data points are marked.

As a more sensitive probe for the detection of self-association of bile salts the fluorescent bile salt derivative 7β-NBD-NCT was used. As the quantum yield of the

TABLE 2. Comparison of physicochemical properties of norcholansulfonate, cholate and cholyltaurine

	t_R[a]	R_f[b]	P_{ow}[c]	CMC[d]	Onset of Self-association[e]
Norcholansulfonate	12.0	0.30	0.31	8.5	~1
Cholate	17.0	0.68	4.75	9.5	~1
Cholyltaurine	12.3	0.27	0.30	7.5	~1

For details see Materials and Methods.

[a] t_R, Retention times of bile salts on a C-18 reversed phase column using methanol–0.01 M potassium phosphate, pH 7.4, as mobile phase.

[b] R_f, Relative mobility on HPTLC using 1-butanol–acetic acid–water 9:2:1 (v/v/v) as mobile phase.

[c] P_{ow}, 1-Octanol/water partition coefficients with 100 mM sodium phosphate, pH 7.4, as aqueous phase.

[d] Determined by dye solubilization using Orange OT as indicator dye with 10 mM phosphate buffer, pH 7.4, adjusted with sodium chloride to an ionic strength of 150 mM.

[e] Determined by fluorescence studies using 7β-NBD-NCT.

fluorescence of 7β-NBD-NCT increases with increasing hydrophobicity of the solvent, raising from 0.025 in water or phosphate buffer, pH 7.4, to 0.398 in 1-octanol (10), gradual association of bile salts could be measured. The dependency of the quantum yields of 5–10 μM 7β-NBD-NCT on the concentration of norcholansulfonate as well as of cholyltaurine and cholate shows that an increase in quantum yields clearly begins at lower concentrations than the apparent CMC values determined by dye solubilization (**Fig. 5**). This dependency allows a distinction between different concentration ranges of association of the examined bile salts: a range of up to about 1 mM exhibiting no significant change in quantum yields, a transient range from about 1 to 6–7 mM characterized by a small, and a range > 6–7 mM showing a stronger increase in quantum yields (Fig. 5). The continual increase in quantum yields of 7β-NBD-NCT with increasing concentrations of all three bile salts shows that an exact indication of CMC values for bile salts is not appropriate. However, as to be expected (13, 34), self-association of bile salts already begins below their respective apparent CMC values.

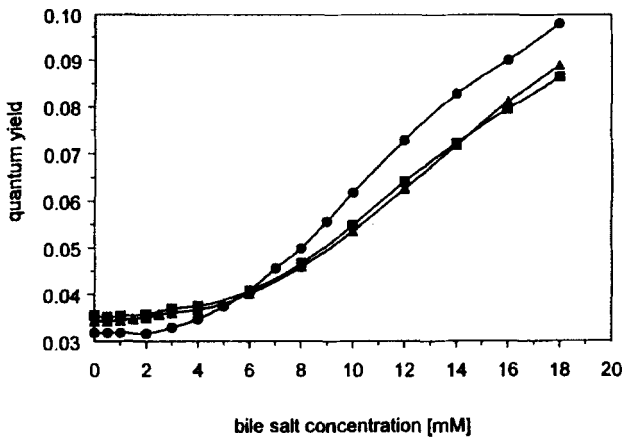

Fig. 5. Effect of norcholansulfonate (▲), cholyltaurine (●), and cholate (■) concentration on quantum yield of the fluorescence of 7β-NBD-NCT (10 μM). Excitation wavelength was 495 nm (see Materials and Methods for details).

Metabolism and excretion of norcholansulfonate

In order to evaluate the suitability of norcholansulfonate for the study of hepatobiliary transport, its overall transport and metabolism in rat liver were studied using the 7β-^{3}H-labeled derivative. Bolus injections of 0.1 to 1 nmol of [7β-^{3}H]norcholansulfonate into a mesenterial vein showed that norcholansulfonate was taken up almost completely in a first pass uptake. It was excreted with a maximum of 3.5 ± 0.5 min (**Fig. 6**), which is comparable to the secretion maximum of cholyltaurine being about 2.5 min. Norcholansulfonate was excreted completely unmetabolized, as analyzed by HPLC and HPTLC. More than 95% of [7β-^{3}H]norcholansulfonate

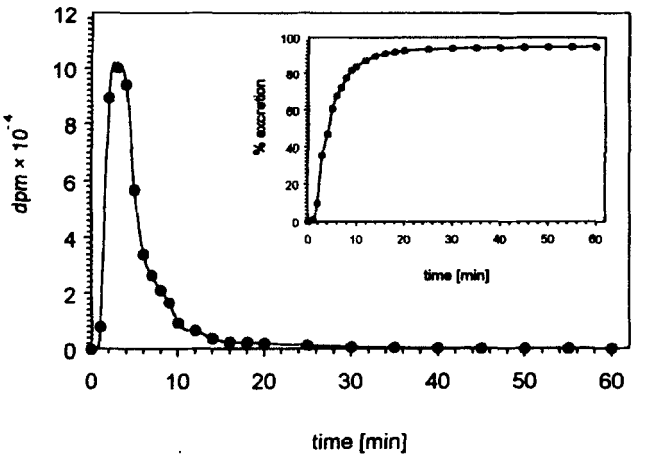

Fig. 6. Time dependency of biliary excretion of [7β-^{3}H]norcholansulfonate (●) after a bolus injection of 1 nmol into a rat mesenterial vein (typical experiment). Bile was collected in 1-min intervals for the first 10 min, in 2-min intervals from 10 min to 20 min, and in 5-min intervals from 20 min to 60 min (n = 6). Radioactivity excreted in the fraction of the respective time. Insert: cumulative % of radioactivity injected.

could be detected in bile within 60 min (insert in Fig. 6), suggesting that removal of tritium from 7β-position by 7α-hydroxysteroid dehydrogenase present in rat liver (35) is of no importance under the experimental conditions used.

The excretion of norcholansulfonate over the path(s) used by the taurine-conjugated bile salts was demonstrated by the mutual inhibition of biliary excretion of norcholansulfonate and secretion of cholyltaurine at the corresponding maximal biliary elimination rates (T_m) of rat liver (**Fig. 7**). Administration of small amounts of norcholansulfonate, which did not increase maximal bile flow resulting from near-saturating conditions incited by constant infusion of 1.6 μmol/(min · 100 g b.w.) cholyltaurine, caused a clear decrease of the secretion rate of cholyltaurine (Fig. 7A). After norcholansulfonate was completely excreted, secretion of cholyltaurine again reached the original secretion rate after 10 min.

In the reverse case, during constant infusion of 1.6 μmol/(min · 100 g b.w.) norcholansulfonate, resulting in nearly the maximal excretion rate (D. Schwab, and G. Kurz, unpublished observations), administration of a small amount of cholyltaurine, which likewise did not alter bile flow, led to a transient decrease in the excretion of norcholansulfonate. After the complete secretion of cholyltaurine, the excretion of norcholansulfonate again reached the original constant value after a period of 10 min (Fig. 7B).

In order to trace whether metabolites were formed from norcholansulfonate at relatively high intracellular concentrations, which will not be reached in intact rat liver because of the fast excretion of the compound, isolated hepatocytes were used to compare the metabo-

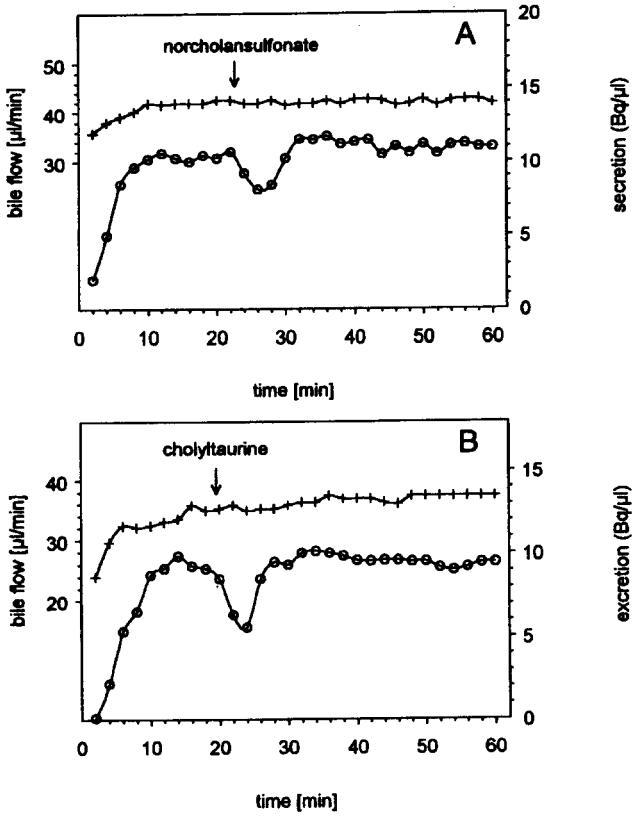

Fig. 7. Effect of norcholansulfonate or cholyltaurine on bile flow (+) and biliary elimination during a constant infusion of the alternative bile salt containing tracer doses of the respective radioactive labeled bile salt. Injection of an additional bolus of the respective non-radioactive labeled bile salt at the time indicated by the arrow. Bile was collected in 2-min intervals. A: Effect of a bolus of non-radioactive labeled norcholansulfonate (5 µmol in 0.5 ml 0.15 M NaCl) during a constant infusion of cholyltaurine (1.6 µmol/(min·100 g b.w.)) containing [G-^{3}H]cholyltaurine (typical experiment). B: Effect of a bolus of non-radioactive labeled cholyltaurine (5 µmol in 0.5 ml 0.15 M NaCl) during a constant infusion of norcholansulfonate (1.6 µmol/(min·100 g b.w.)) containing [7β-^{3}H]norcholansulfonate (typical experiment).

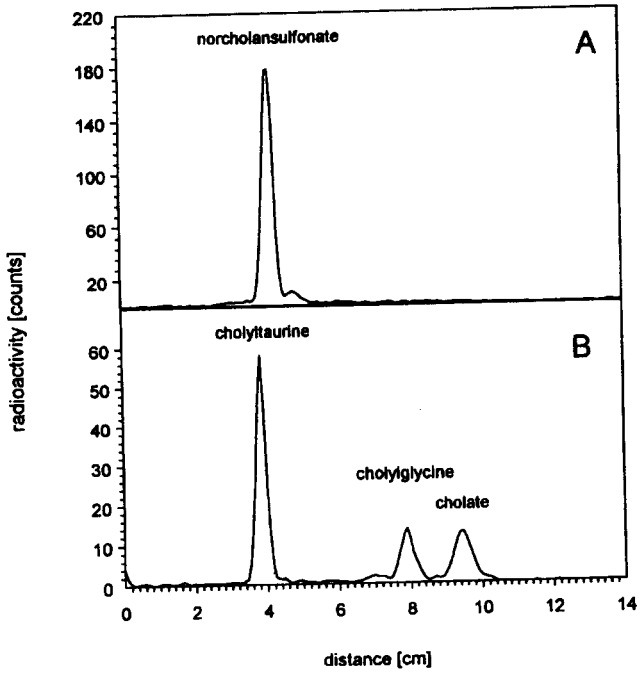

Fig. 8. Comparison of metabolism of norcholansulfonate and cholate in isolated hepatocytes. Suspensions of 1×10^6 to 2×10^6 isolated hepatocytes per ml were incubated with 5 µM of the respective bile salt for 10 min. After centrifugation of hepatocytes and methanolic extraction of the resulting pellet, extracts were separated by HPTLC in solvent system 1-butanol–acetic acid–water 9:2:1 (v/v/v). Radioactivity was detected with the aid of a linear radiochromatogram scanner (see Materials and Methods for details). A: Radiochromatogram of metabolites of norcholansulfonate. B: Radiochromatogram of metabolites of cholate.

lism of norcholansulfonate with that of cholate. For this, suspensions of isolated hepatocytes were incubated for different lengths of time with 5 to 100 µM norcholansulfonate or cholate. Within 10 min norcholansulfonate was transformed to varying extents, not exceeding 5%, to a less polar metabolite which was found predominantly within the cells (**Fig. 8A**) and only to a small amount in the incubation medium. As to be expected, under the same experimental conditions, cholate was mainly conjugated to cholyltaurine and cholylglycine, whereas other metabolites were found only in negligible amounts. The conjugated bile salts formed were likewise found to the greater part within the cells (Fig. 8B) and to the smaller part in the medium. Within the time of maximally 1 min needed for transport studies, neither metabolites of norcholansulfonate nor of cholate could be detected in the incubation medium.

DISCUSSION

In order to make the accessibility of C-23 bile salt sulfonates easy, we used the Kochi reaction (31), which simultaneously allows the degradation of the side chain of cholic acid by one C-atom and the introduction of the chlorine substituent as a good leaving group (29). Compared with the recently described multistep procedure (36), this reaction offers a direct and simple way to synthesize bile salt sulfonates. Synthesis of radioactive labeled derivatives was performed by the well-established introduction of tritium in the 7β-position. Reduction of the 7-oxo derivatives of norcholansulfonate, as well as that of cholate, resulted in the formation of 90% of the 7β-^{3}H isomers, as proved by cochromatography and 10% of an isomer probably with the 7α-^{3}H configuration. The ratio of the diastereomers is comparable to that obtained by the reduction of other 7-oxo steroids with complex hydrides (27, 37, 38).

For biological comparison of norcholansulfonate with the physiological bile salts cholyltaurine and cholate, it must be shown that they do not differ in physicochemical properties relevant to the study of mediated membrane transport. A characteristic property of a bile salt is its hydrophobic–hydrophilic balance (12, 39, 40). Therefore, the reversed phase HPLC mobility of norcholansulfonate was compared to that of cholyltaurine and cholate. Due to the fact that hydrophobic–hydrophilic properties are strongly influenced by the charge of the molecule, an apparent pH value of 7.4 was chosen, to ensure complete ionization of all bile acids. The retention times on a C-18 reversed phase column of norcholansulfonate and cholyltaurine are similar but differ from that of cholate (Table 2). The similarity of norcholansulfonate and cholyltaurine and their difference from cholate were also manifested by their relative retention times in HPTLC (Table 2). The difference between the bile salt sulfonates on the one hand and the bile salt carboxylate on the other hand is clearly confirmed by the corresponding 1-octanol/water coefficients (Table 2). With regard to the relative balance of hydrophobic–hydrophilic properties, norcholansulfonate and cholyltaurine are similar and differ considerably from cholate.

The tendency of bile salts to associate in aqueous solutions with increasing concentrations limits the concentration range useful for kinetic studies. Therefore, self-association of the corresponding bile salts was studied by dye solubilization (19), and by fluorescence enhancement of a fluorescent bile salt derivative. As Orange OT is in common use for CMC determination of bile salts, the change of its absorbance was followed in dependency on bile salt concentration. The determined apparent CMC values of all three bile salts differed only a little, with that of norcholansulfonate lying between the values of cholyltaurine and cholate (Table 2). It must be noted that the CMC of cholate presented in this paper is lower than that determined previously by the same method (19).

As the detection of the extent of association of bile salts is dependent on the method applied, a more sensitive method than dye solubilization was attempted to determine the early stages of association. For this purpose spectral changes in fluorescence emission were investigated using the fluorescent bile salt derivative 7β-NBD-NCT as a sensitive probe. This derivative bears the relatively small NBD-amino fluorophore at position 7 of the steroid nucleus and does not basically differ in its hydrophobic–hydrophilic properties from cholyltaurine (14). As a compound soluble in aqueous solutions, solubilization prior to interaction with a bile salt is not required, and therefore the influence of the probe on self-association of the added bile salt should be minimal. The optical properties of the NBD-amino group are sensitive to the hydrophobicity of its environment (10, 14–16). Decreasing polarity of the solvent leads to higher quantum yields and to a shift of the emission maxima to shorter wavelengths. Association of bile salts should result in an increase in the hydrophobicity of the environment of the NBD-amino group, and consequently to an increase in quantum yield and a shift of emission maxima. Whereas the shift of emission maxima of 7β-NBD-NCT with increasing bile salt concentrations did not allow precise analysis due to the broad width of the emission band, the increase in quantum yields made clear analysis possible. The increase in quantum yields of 7β-NBD-NCT did not begin until each of the examined bile salts had reached a concentration of about 1 mM (Fig. 5). Up to this concentration the examined bile salts must be assumed to exist, under experimental conditions, in the monomeric form.

The dependencies of quantum yields from bile salt concentrations show that the increase in hydrophobicity occurs over a broad concentration range. This emphasizes that association of bile salts takes place gradually, and consequently, there were no sharp points of micelle formation, as suggested by dye solubilization. The correspondence between the two bile salts with the shorter side chain and cholyltaurine demonstrates that they do not differ considerably in their association behavior.

Norcholansulfonate was not expected to be transformed to taurine or glycine conjugates. However, because the formation of other conjugates could not be ruled out in advance, the behavior of norcholansulfonate and the metabolic stability of the tritium-label in the 7β-position were investigated under the conditions used for transport studies. In rat liver [7β-^{3}H]norcholansulfonate was completely excreted unmetabolized with an excretion maximum similar to the secretion maximum of cholyltaurine (Fig. 6). Recovery of [7β-^{3}H]norcholansulfonate exceeded 95% within 60 min, suggesting that loss of tritium by oxidative reactions is practically without relevance. Biliary excretion of norcholansulfonate was inhibited by cholyltaurine, and vice versa secretion of cholyltaurine was inhibited by norcholansulfonate (Fig. 7), indicating that both bile salts share the same path(s) of bile salt secretion.

Whereas in intact liver norcholansulfonate excretion guarantees that the intracellular concentrations do not reach such levels that metabolic processes might play a role, in isolated hepatocytes concentrations may be achieved that could result in metabolic transformations. In isolated hepatocytes norcholansulfonate was metabolized within 10 min to about 5% to a metabolite, the structure of which has not yet been elucidated, whereas cholate was mainly conjugated to cholyltaurine and cholylglycine (Fig. 8). Within the time necessary for

uptake studies, this metabolite of norcholansulfonate could not be detected in the incubation medium and only in insignificant traces within the hepatocytes.

Regarding metabolism and biliary excretion in intact rat liver, as well as in freshly isolated hepatocytes, norcholansulfonate behaved as a physiological bile salt. The physicochemical and metabolic properties of norcholansulfonate make it a suitable analogue of cholate for the study of hepatobiliary transport of unconjugated C-24 bile salts.

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