papers and notes on methodology

3-Diazirine-derivatives of bile salts for photoaffinity labeling

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Abstract New carbene-generating photolabile bile salt derivatives, 3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oic acid and (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid were synthesized with high specific radioactivity. These 3-diazirine-derivatives could be activated to the corresponding carbenes by irradiation with ultraviolet light at 350 nm with a half-life time of 2 min. The 3-diazirine derivatives behaved in enterohepatic circulation like the natural bile salts. The uptake of [^{3}H]taurocholate into isolated hepatocytes was competitively inhibited by (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid indicating that the 3,3-azo-derivative of taurocholate shares the hepatic transport systems for natural bile salts. It was demonstrated that the radioactively labeled 3-diazirine bile salt derivatives are useful probes for photoaffinity labeling of bile salt binding proteins especially in intact cells and tissues.—**Kramer, W., and S. Schneider.** 3-Diazirine-derivatives of bile salts for photoaffinity labeling. *J. Lipid Res.* 1989. **30:** 1281–1288.

Supplementary key words Hepatocytes • carbene-generating photolabile bile salts

Photolabile bile salt derivatives (1) have been successfully used for the identification of bile salt transport systems in blood (2), liver (3–7), small intestine (8,9), and kidney (10). The labeling pattern of the respective bile salt binding polypeptides in the different tissues is dependent upon the chemical structure of the utilized derivative. These differences are caused by the different reactivities of the photogenerated carbenes and nitrenes and the position of the photoreactive group within the bile salt molecule. Therefore, a set of different carbene- and nitrene-generating photolabile derivatives with the photolabile groups at different positions of the bile salt molecule is necessary to ensure that no bile salt-binding protein will escape detection. The 7-diazirino-bile salt derivatives have short half-life times under irradiation at 350 nm of about 2 min, compared to 8.5–18 min for the 3- and 11-azido-derivatives upon irradiation at 300 nm (1). Due to their short half-life time upon photolysis the diazirine-derivatives are especially suited for photoaffinity labeling of intact cells and tissues (6,11,12), because short irradiation times are desirable to avoid photolytic damage of cells. In the present study we describe the synthesis of new diazirine-derivatives of bile salts with the photolabile diazirino-group attached at position 3 of the steroid nucleus.

EXPERIMENTAL

Materials

Cholic acid, taurine, silica gel N 60 (40–63 μm) and silica plates for analytic and preparative thin-layer chromatography were obtained from Merck (Darmstadt, F. R. G.). Sodium boro[^{3}H]hydride (5–10 Ci/mmol) was obtained from Amersham Buchler GmbH (Braunschweig, F. R. G.). All other chemicals were purchased in best quality from the usual commercial sources.

Methods

Elemental analyses were carried out with a Perkin-Elmer 240 analyzer (Perkin-Elmer, Friedrichshafen, F. R. G.). ^{1}H-NMR-spectra were recorded on a Bruker 250 MHz spectrometer Spectrospin WM 250 (Bruker Physics, Karlsruhe, F. R. G.). Values are given in parts per million relative to tetramethylsilane as internal standard. Mass spectra were measured with a Finnigan MAT 445 (Finnigan MAT GmbH, Bremen, F. R. G.) mass spectrometer. Photolyses were carried out in a Rayonet photochemical reactor RPR 100 (The Southern Ultraviolet Company, Hamden, Conn., USA) equipped with 16 RPR 3500 Å lamps using the cuvettes and techniques described (2,4, 11). Bile acid derivatives were detected on thin-layer chro-

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matograms by spraying the plates with conc. sulfuric acid and then heating at 80°C for 5 min.

Hepatocytes were isolated according to Berry and Friend (13). Uptake studies into isolated hepatocytes were performed by the rapid centrifugation method as described (14,15). For perfusion studies the respective bile salt derivatives (1–2 μCi dissolved in 200 μl of 0.9% sodium chloride solution) were injected into the upper mesenterial vein of anesthesized rats and bile was collected from the cannulated common bile duct. Radioactivity in bile samples was determined by liquid scintillation counting. The composition of bile salts in the bile samples was analyzed by thin-layer chromatography and subsequent radiochromatography scanning.

For photoaffinity labeling freshly prepared hepatocytes (1–2 $\times$ 10^6 hepatocytes in 1–2 ml of 118 mM sodium/potassium phosphate buffer (pH 7.4), 4.74 mM KCl, 24.87 mM $NaHCO_3$, 1.185 mM $MgCl_2$, 1.25 mM $CaCl_2$, 5.5 mM D-glucose were incubated for 1 min with radioactively labeled photoreactive bile salt derivatives in the dark and subsequently photolyzed for 5 min at a wavelength of 350 nm in a Rayonet RPR-100 photochemical reactor. After irradiation the cells were diluted with 10 mM sodium phosphate buffer (pH 7.4), and after a further 10-min incubation the osmotically shocked cells were centrifuged at 15,000 *g* for 30 min. For analysis of incorporated radioactivity, the resulting pellets were dissolved in 100 μl of 62.5 mM Tris-HCl buffer (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and polypeptides were separated by SDS gel electrophoresis on slab gels (180 $\times$ 240 $\times$ 1.5 mm) (9). After fixing and staining, the gels were scanned and the distribution of radioactivity was determined either by fluorography (16) with sodium salicylate as scintillator or by liquid scintillation counting of the gels after slicing of the gel tracks into 2-mm pieces and digestion of proteins with Biolute (Zinsser Analytic GmbH, Frankfurt/F. R. G.).

Syntheses

The solvent systems used for chromatographic separations were: solvent system 1: ethylacetate–cyclohexane–acetic acid 23:7:3; solvent system 2: ethylacetate– cyclohexane–acetic acid 100:40:1; solvent system 3: ethylacetate–cyclohexane–acetic acid 200:20:1; solvent system 4: n-butanol–acetic acid–water 9:2:1.

3α-Hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid

Two g (4.05 mmol) of 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid (17) was dissolved in 20 ml of dry methanol. Five ml of a 1 M methanolic solution of potassium hydroxide was added and after 8 min of vigorous stirring at room temperature the reaction mixture was poured into 400 ml of 1 M HCl. The precipitate was collected by filtration yielding 1.8 g (3.8 mmol) of 3α-hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid, which was recrystallized from ethanol/water. Yield: 93.8%; mp: 180–181°C. TLC: R_f = 0.66 (solvent system 1), 0.25 (solvent system 2), 0.24 (solvent system 3); ^{1}H-NMR ($CDCl_3$): δ = 0.75 (s, C$\underline{H}_3$-18), 0.85 (d, C$\underline{H}_3$-21, J = 6 Hz), 0.93 (s, C$\underline{H}_3$-19), 3.53 (m, C$\underline{H}$-3), 5.06 (s, C$\underline{H}$-OCHO-7), 5.28 (s, C$\underline{H}$OCHO-12), 8.1 (s, CH-OC$\underline{H}$O-7), 8.15 (s, CH-OC$\underline{H}$O-12). Anal. calcd. for $C_{26}H_{40}O_7$ (464.6): C, 67.22, H, 8.68, found: C, 67.14, H, 8.75

3-Oxo-7α,12α-diformyloxy-5β-cholan-24-oic acid

3α-Hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid (3.72 g, 8 mmol) was dissolved in 400 ml of 80% aqueous acetone and subsequently 1.7 equivalents (13.6 mmol, 2.42 g) of solid N-bromosuccinimide were added. The solution immediately became orange and after about 2 h the orange color disappeared. Thin-layer chromatographic monitoring using solvent system 2 revealed that the reaction was complete. The solution was concentrated by evaporation to about 50 ml and after addition of 20 ml of dimethylsulfoxide the bile acid was precipitated from 500 ml of 1 M HCl. Yield: 3.6 g (7.8 mmol), 97.5%. The compound was recrystallized from ethanol/water. mp: 160–162°C. TLC: R_f = 0.73 (solvent system 1), 0.42 (solvent system 2), 0.32 (solvent system 3). ^{1}H-NMR ($CDCl_3$): δ = 0.81 (s, C$\underline{H}_3$-18), 0.87 (d, C$\underline{H}_3$-21, J = 6 Hz), 1.05 (s, C$\underline{H}_3$-19), 3.02 (C$\underline{H}$-2 or C$\underline{H}$-4, J = 15 Hz), 5.16 (s, C$\underline{H}$-OCHO-7), 5.32 (s, C$\underline{H}$-OCHO-12), 8.10 (s, CH- OC$\underline{H}$O-7), 8.16 (s, CH-OC$\underline{H}$O-12). Anal. calcd. for $C_{26}H_{38}O_7$ (462.5): C, 67.52, H, 8.28. Found: C, 66.94, H, 8.32

3-Oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid

3-Oxo-7α,12α-diformyloxy-5β-cholan-24-oic acid (2.3 g, 5 mmol) was dissolved in 70 ml of a 1 M methanolic solution of potassium hydroxide at 25°C. After 1–2 h the formyl groups had been removed as monitored by thin-layer chromatography in solvent system 1. The product was precipitated from 500 ml 1 M HCl and recrystallized from ethanol/water yielding 1.78 g (4.41 mmol) of pure 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid. Yield: 88.2%; mp: 174–175°C. TLC: R_f = 0.50 (solvent system 1), 0.12 (solvent system 2), 0.13 (solvent system 3). ^{1}H-NMR ($CDCl_3$): δ = 0.72 (s, C$\underline{H}_3$-18), 0.87 (d, C$\underline{H}_3$-21, J = 6 Hz), 0.98 (s, C$\underline{H}_3$-19), 3.39 (C$\underline{H}$-2 or C$\underline{H}$-4, J = 15 Hz), 3.92 (s, C$\underline{H}$-7), 4.05 (s, C$\underline{H}$-12). Anal. calcd. for $C_{24}H_{38}O_5$ (406.6): C, 70.89, H, 9.42. Found: C, 70.44, H, 9.82

3,3-Azo-7α12α-dihydroxy-5β-cholan-24-oic acid

3-Oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid (1.1 g, 2.7 mmol) was dissolved in 70 ml of freshly prepared abso-

lute methanol and dry ammonia was bubbled through the solution at – 10°C for 10 h. Subsequently a solution of 2 g (18 mmol) of hydroxylamine-O-sulfonic acid in 20 ml of absolute methanol was added during 30 min. The mixture was allowed to warm up and was subsequently stirred for 20 h. After filtration 1 ml of N-triethylamine was added and the solution was evaporated to dryness. The residue was redissolved in 30 ml of absolute methanol and after addition of 1 ml of N-triethylamine solid iodine was added in 50-mg portions until the brown color persisted. The reaction mixture was evaporated to dryness, the residue was dissolved in 20 ml of dimethylsulfoxide, and the crude diazirine was precipitated from 400 ml of 1 M HCl. The product was dried and dissolved in 25 ml of ethylacetate–cyclohexane–acetic acid 100:40:1 (v/v/v) and purified by flash chromatography (18) on a 30 × 3 cm column of silica gel N 60 using the aforementioned solvent as eluent. The fractions containing the pure 3-diazirine were kept at – 20°C for 10 h. Three hundred fifty mg of pure 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was obtained as white crystalline needles and another 260 mg was obtained from the mother liquor by evaporation. Yield: 54%; mp: 162–164°C (decomposition). TLC: R_f = 0.68 (solvent system 1), 0.38 (solvent system 2), 0.37 (solvent system 3). UV (methanol): λ_{max} = 370 nm (ϵ = 55), 352 nm (ϵ = 62). Mass spectrum: m/e 372 ($M^+-N_2-H_2O$), m/e 354 ($M^+-N_2-2\ H_2O$), m/e 253 ($M^+-\ N_2-2\ H_2O$–side chain with m/e 101, base peak). ^{1}H-NMR ($CDCl_3/CD_3OD$ = 3/1, v/v): δ = 0.73 (s, C$\underline{H}_3$–18), 1.00 (d, C$\underline{H}_3$–21, J = 6 Hz), 1.02 (s, C$\underline{H}_3$–19), 2.91 (C$\underline{H}$–2 or C$\underline{H}$–4, J = 15 Hz), 3.84 (s, C$\underline{H}$–7), 3.97 (s, C$\underline{H}$–12). Anal. calcd. for $C_{24}H_{38}N_2O_4$ (418.56): C, 68.86, H, 9.15, N, 6.69 found: C, 68.25, H, 9.10, N, 6.31.

The synthesis of (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid was performed via the mixed anhydride method as previously described (1,19) starting from 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid.

Synthesis of 3,3-azo-7α,12α-dihydroxy-5β-[7β-^{3}H]cholan-24-oic acid and (3,3-azo-7α, 12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid

Two hundred nine mg (0.5 mmol) of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was dissolved in 20 ml of 80% aqueous acetone and subsequently 140 mg (0.75 mmol) of N-bromosuccinimide was added. After 2–4 h the solution was evaporated to dryness at 30°C. The crude product was purified by flash chromatography (18) on a 15 × 3 cm column of silica gel N 60 using ethylacetate–cyclohexane–acetic acid 100:40:1 (v/v/v) as solvent. One hundred fifty-five mg (0.37 mmol) of 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-24-oic acid (R_f = 0.57, solvent system 2) and 49 mg (0.12 mmol) of 3,3-azo-7-oxo-12-oxo-5β-cholan-24-oic acid (R_f = 0.68 in solvent system 2) were obtained. (3,3-Azo-7-oxo-12α- hydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid was synthesized from 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-24-oic acid as described (1).

For the synthesis of radiolabeled 3-diazirine derivatives of bile salts in unconjugated and taurine-conjugated form, 0.02 mmol of 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-24-oic acid or its taurine conjugate was dissolved in 300 μl of dioxane and 30 μl of 1 M aqueous sodium hydroxide. This solution was added to 100 mCi of sodium boro[^{3}H]hydride (5.2 Ci/mmol). After 12 h at 20°C in the dark, 50 μl of 2 M HCl was added and after a further 2 h the reaction mixture was put on preparative high performance thin-layer chromatography plates (20 cm × 5 cm × 1 mm). The chromatograms were developed in solvent system 2 for the unconjugated and solvent system 4 for the taurine-conjugated derivatives. The compounds were extracted as described (1) yielding 10–15 mCi of the corresponding photolabile salt derivatives with a specific radioactivity of 1.3 Ci/mmol.

RESULTS

Synthesis of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid

For the synthesis of 3,3-azo-derivatives of bile salts, the corresponding 3-oxo-compounds are necessary as starting material. 3-Oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid has been synthesized from cholic acid methylester by Oppenauer-oxidation (20) or by oxidation with silver carbonate on Celite (21,22) and subsequent alkaline hydrolysis of the resulting 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid methylester. However, during alkaline hydrolysis of the 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid methylester obtained with these procedures, the 3-oxo-group was destroyed and only tiny amounts (< 5%) of 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid could be isolated after chromatography; this vulnerability of the 3-oxo group in bile acids during alkaline conditions was also found by others (23). Therefore, 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid was synthesized by a route similar to that of Leppick (24). Triformyloxycholic acid was selectively deformylated at position 3 to give 3-hydroxy-7α,12α- diformyloxy-5β-cholan-24-oic acid, which was oxidized to 3-oxo-7α,12α-diformyloxy-5β-cholan-24-oic acid with N-bromosuccinimide in 80% aqueous acetone. The remaining formyl groups in positions 7 and 12 could be removed under mild alkaline conditions at room temperature without affecting the 3-oxo-function.

3-Oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid was transformed to the 3-diazirine-derivative by reaction with hydroxylamine-O-sulfonic acid in methanolic ammonia so-

lution and subsequent oxidation of the 3-diaziridine with iodine as described (1,25). In contrast to the synthesis of 7,7-azo-derivatives of bile salts, this reaction step from the 3-oxo to the 3-diazirine-derivatives (1) is extremely sensitive to moisture. The formation of the 3-diazirine occurred only with absolutely dry compounds and solvents. The 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was characterized by elemental analysis, UV, NMR, and mass spectrometry. 3,3-Azo-7α,12α-dihydroxy-5β-cholan-24-oic acid shows the typical ultraviolet absorption of the diazirino-function with a doublet at 370 and 352 nm (1,25,26). The NMR-spectrum proved the structure of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid; the typical signals of the H-atoms at position 7 (δ = 3.84) and position 12(δ = 3.97) were present, whereas no proton signal at δ = 3.30 was observed, since the 3α-hydroxyl group and the H-atom at 3β-position are replaced by the 3,3-azo-function. As with the 7,7-azo-isomere (1) no molecular ion peak at m/e 418 could be detected in the mass spectrum of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid. The fragmentation pattern was very similar to that of the 7-isomer with loss of N_2 and H_2O molecules (fragments m/e 372 and 354) and of the side chain (m/e 101) leading to the base peak m/e 253. Photolysis of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid at 350 nm in a Rayonet photochemical reactor revealed a half-life time about 2 min under the conditions used (**Fig. 1**).

For the preparation of radioactively labeled 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid and its taurine conjugate, the 7α-hydroxy group was oxidized with N-bromosuccinimide in 80% aqueous acetone to give the corresponding 7-oxo-derivatives. Radioactivity was introduced by reduction of the 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-24-oic acid and its taurine conjugate with sodium boro[^{3}H]hydride in dioxane water (1). The reduction of 7-oxo-derivatives of bile salts with sodium borohydride leads nearly completely to the 7α-hydroxy-derivative (27,28). After reduction of 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-24-oic acid with sodium boro[^{3}H]hydride, only one radioactively labeled band comigrating with 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was detectable after thin-layer chromatography of the reaction mixture in different solvent systems. A further confirmation of the structure of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was given by control experiments where 7-oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid and 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-24-oic acid were reduced with sodium borohydride. After purification of the reaction products by preparative thin-layer chromatography, the signal of the H-atom at position 7 in the ^{1}H-NMR-spectra was observed at δ = 3.84 indicating an equitorial orientation of the H-atom and an axial configuration of the 7-hydroxy-group (i.e. α-position); a 7β-hydroxyl-derivative would have given a signal of the 7-H atom at δ = 3.58 (28). Therefore, reduction of 3,3-azo-7-oxo-derivatives of bile salts with Na[^{3}H]BH_4 yields photoreactive bile salt derivatives with α-orientation of the hydroxyl groups at positions 7 and 12 as in the natural bile salts. Photoaffinity labeling experiments with hepatocyte membranes using the 12α-hydroxy and the 12β-hydroxy-derivatives of 3β-azido- and 7,7-azo-derivatives of cholate and taurocholate revealed no significant differences in the labeling pattern (W. Kramer, unpublished results).

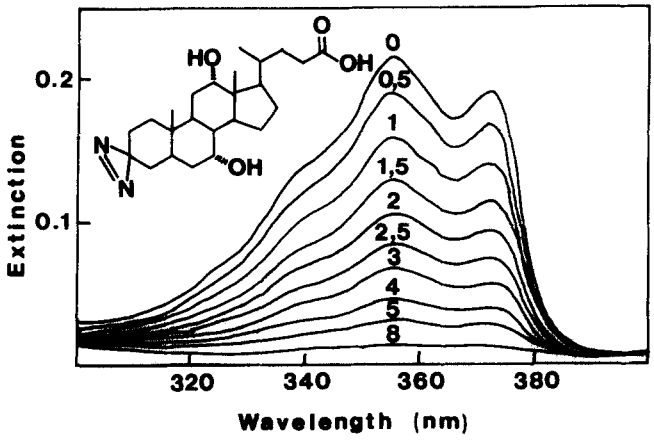

Fig. 1. Photolysis of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid. A solution of 1.14 mg of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid in 1 ml of methanol was photolyzed in a Rayonet RPR 100 photochemical reactor equipped with 16 RPR 3500 Å lamps for 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 8 min. The absorption of the diazirino group was recorded after the respective times of photolysis.

Photoaffinity labeling

In order to determine whether the new photolabile 3,3-azo-derivatives of bile salts behave like the natural bile salts, [^{14}C]taurocholate and (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid were simultaneously injected into the superior mesenteric vein of an anesthesized rat after cannulation of the common bile duct. Both bile acids appear in bile with a maximum of secretion of taurocholate at 4 min and at 6 min for (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid (**Fig. 2**). During a bile collection period of 60 min, 87% of (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid and 93% of [^{14}C]taurocholate appeared in bile. The slightly retarded excretion of bile salts without a 3-hydroxyl group was formerly observed with 3β-azido-derivatives of taurocholate (29,30). After installation of (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid into the lumen of the terminal ileum, the compound also appeared in bile, indicating that this substance is also absorbed from the small intestine similar to the natural bile salts. Thin-layer chromatography of the respective bile samples revealed that the 3,3-azo-derivative of taurocholate underwent the enterhepatic circulation without metabolic

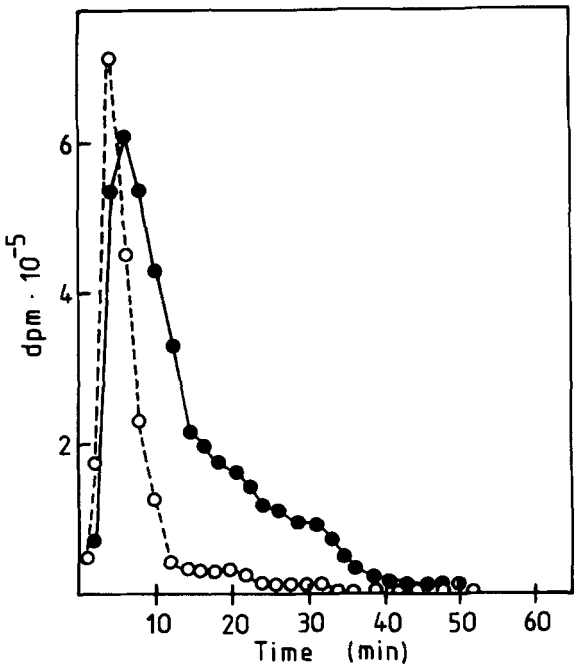

Fig. 2. Biliary excretion of (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid and [^{14}C]taurocholate. Two μCi of (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid (●) and 1 μCi [^{14}C]taurocholate (○) dissolved in 200 μl 0.9% sodium chloride solution was injected into the superior mesenteric vein of an anesthesized rat. Bile was collected at 2-min intervals and radioactivity was determined by liquid scintillation counting.

transformation like taurocholate. **Fig. 3** shows that the Na^+-dependent uptake of [^{3}H]taurocholate into isolated hepatocytes was competitively inhibited by (3,3-azo-7α, 12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid, indicating that the photoreactive derivative shares common uptake systems with taurocholate.

With photolabile bile salt derivatives carrying nitrene-generating aliphatic azido groups at positions 3 or 11 and a carbene-generating diazirino group at position 7, bile salt-binding proteins in different tissues have been characterized (2-12,29,31). Due to their short half-life time of about 2 min compared to 8-18 min for the azido-derivatives (1,29) the 7,7-azo-derivatives are especially suited for the labeling of intact cells and living tissue (11,12). In sinusoidal surfaces of hepatocytes, two polypeptides of molecular weights 54,000 and 48,000 were identified as putative bile salt transport systems (3-6). The 54,000 molecular weight polypeptide was predominantly labeled with the (7,7-azo-3α-12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid, whereas the 3- and 11-azido derivatives led to highest labeling of the polypeptide of molecular weight 48,000. Similar differences in the labeling pattern of bile salt-binding proteins by 7-diazirine and 3- or 11-azido bile salt derivatives were observed in the small intestine (9) and in blood (2).

In order to determine whether the different chemical reactivities of carbenes and nitrenes or the position of the photolabile group within the bile salt molecule are predominantly responsible for these differences in the labeling pattern, the labeling of bile salt-binding polypeptides in isolated hepatocytes was chosen as an example to demonstrate that by use of only one photolabile substrate analogue some binding polypeptides may escape detection. For the identification of bile salt-binding polypeptides in hepatocyte membranes, freshly prepared isolated hepatocytes were submitted to photoaffinity labeling with 3,3-azo- and 7,7-azo-derivatives of taurocholate. After photoaffinity labeling the cells were washed with hypotonic medium, and after separation from soluble proteins by centrifugation the resulting pellets containing cell organelles were submitted to SDS gel electrophoresis.

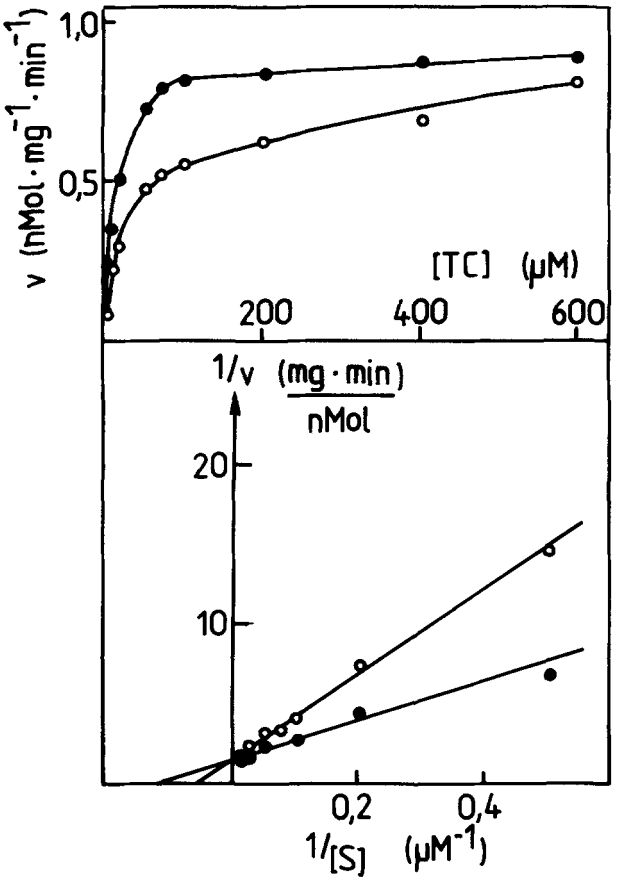

Fig. 3. The effect of (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid on the Na^+-dependent uptake of [^{3}H]taurocholate into isolated hepatocytes. Freshly prepared isolated hepatocytes in 118 mM sodim/potassium phosphate buffer (pH 7.4), 4.74 mM KCl, 24.87 mM $NaHCO_3$, 1.185 mM $MgCl_2$, 1.25 mM $CaCl_2$, 5.5 mM D-glucose were incubated at 37°C with the indicated concentrations of [^{3}H]-taurocholate in the absence (●) and in the presence (○) of 100 μM (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid. Under the conditions used the inhibition constant K_I was 53 μM. [TC]: concentration of taurocholate.

Fig. 4A shows a fluorogram of these fractions after photoaffinity labeling of hepatocytes either with (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid (track a) or (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid (track b). With the 7,7-azo-derivative predominantly the bile salt-binding polypeptide of molecular weight 54,000 of the hepatocyte membrane was labeled, whereas the membrane polypeptide of molecular weight 48,000 was only slightly labeled; a predominant predominant labeling of the mitochondrial polypeptide of molecular weight 33,000 was also found. Minor labeled bands with apparent molecular weights of 100,000, 95,000, 75,000, 67,000, and 24,000 were also detected. In contrast, labeling of the same hepatocyte preparation with the 3,3-azo-isomere resulted in a markedly different labeling pattern. (3,3-Azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-amino-ethanesulfonic acid led to a predominant labeling of the Na^+-dependent bile salt carrier protein of molecular weight 48,000–49,000 (3,4, 32); the bile salt-binding membrane protein of molecular weight 54,000—the predominantly labeled polypeptide by the 7,7-azo-derivative of taurocholate and assumed to be a component of the Na^+-independent bile salt transport system (31)—was labeled to a much lesser extent than the polypeptide of molecular weight 48,000. The mitochondrial polypeptide of molecular weight 33,000 was hardly labeled whereas predominant labeling of a hitherto unidentified bile salt binding polypeptide of molecular weight 24,000 occurred. Labeled polypeptides with apparent molecular weights of 100,000, 95,000, 75,000, and 67,000 were also detected.

In order to evaluate the specificity of the labeled polypeptides for bile salts, competition photoaffinity labeling experiments of isolated hepatocytes in the presence of un-

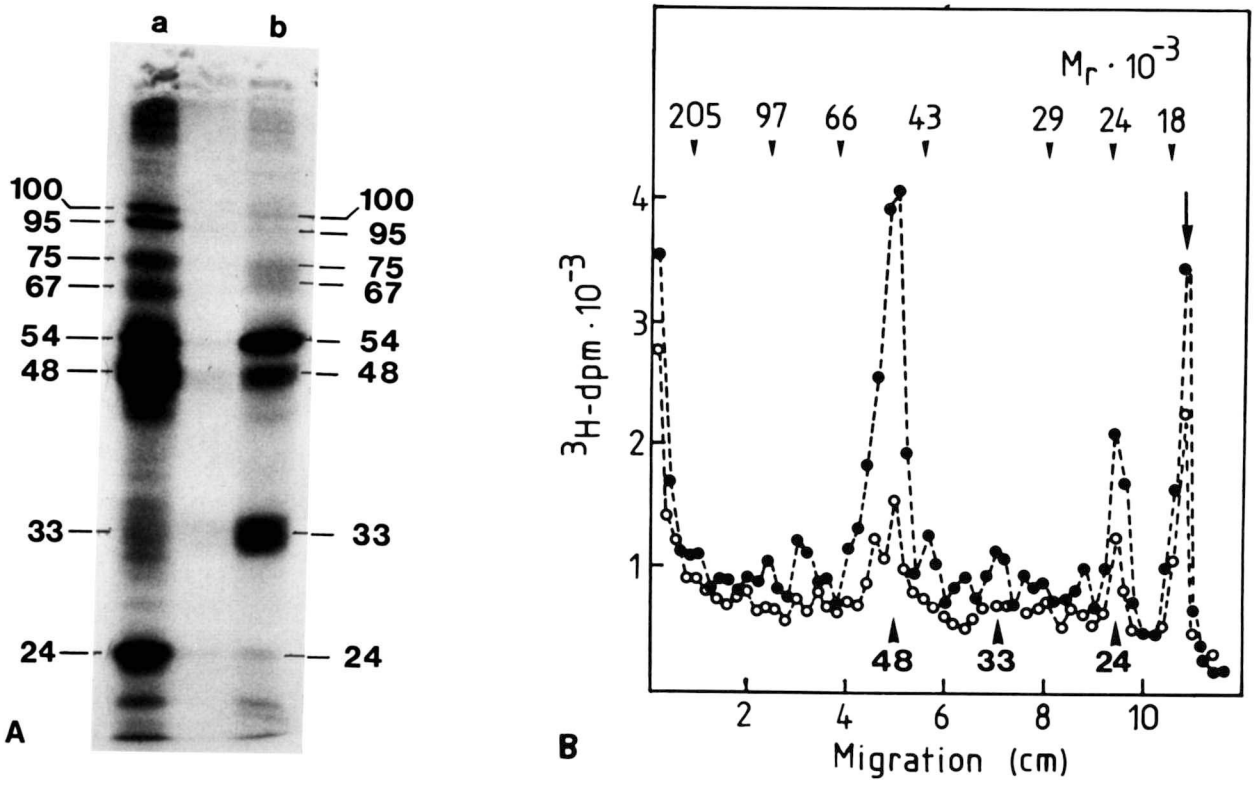

Fig. 4. Distribution of radioactivity after sodium dodecylsulfate polyacrylamide gel electrophoresis of isolated hepatocytes photolabeled with diazirine derivatives of bile salts. A: Freshly prepared hepatocytes (2×10^6) suspended in 1 ml of buffer were incubated at 37°C for 2 min either with 1.25 μM (25 μCi) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid or 19 μM (25 μCi) (3,3-azo-7α,12α-dihydroxy- 5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid and then photolyzed at 350 nm for 5 min. After washing the hepatocytes were suspended in 10 mM sodium phosphate buffer, pH 7.4. After centrifugation at 15,000 *g*, the resulting pellets containing cell organelles were submitted to sodium dodecylsulfate polyacrylamide gel electrophoresis on 9% gels (9). The gels were dried and submitted to fluorography (16) for 2 weeks at −80°C. The numbers indicate the molecular weights $\times 10^{-3}$ of the bile salt-binding polypeptides. Track a: Labeling with (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid. Track b: Labeling with (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid. B: Isolated hepatocytes (1×10^6) suspended in 1 ml of buffer were incubated at 37°C for 2 min in the dark with 19 μM (25 μCi) (3,3-azo-7α, 12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid either in the absence (●) or in the presence (○) of 1 mM taurocholate and subsequently submitted to photoaffinity labeling. After washing of the cells the hepatocytes were resuspended in 10 mM sodium phosphate buffer. After centrifugation at 15,000 *g* the resulting pellets containing cell organelles were submitted to SDS gel electrophoresis on 10.5% gels. The distribution of radioactivity was determined by liquid scintillation counting after slicing of the gels into 2-mm pieces.

labeled taurocholate were performed either with the 3,3-azo- or the 7,7-azo-derivatives of taurocholate (Fig. 4B). For detection of radioactivity in the labeled polypeptides both fluorography and liquid scintillation counting of the gels after slicing were used. The presence of unlabeled taurocholate predominantly reduced the labeling of the bile salt-binding proteins having molecular weights of 48,000 and 54,000 independently whether the 3,3-azo- or the 7,7-azo-derivative of taurocholate was used as photoprobe; the labeling of the polypeptides of molecular weight 67,000 (identified as membrane-bound albumin (4)) and 75,000 (presumably proalbumin as indicated from immunoprecipitation experiments) was also reduced by taurocholate. The labeling of the polypeptides of molecular weights 95,000 and 100,000, the latter possibly being the canalicular bile acid carrier (33), was prevented by taurocholate. The extent of labeling of the mitochondrial polypeptide of molecular weight 33,000, which was the predominantly labeled polypeptide when the 7,7-azo-derivative was used, was also decreased by taurocholate, but to a lesser extent than the abovementioned polypeptides. The labeling of the hitherto unidentified polypeptide of molecular weight 24,000 which was very strongly labeled by (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]-cholan-24-oyl)-2-aminoethanesulfonic acid, was decreased by the presence of taurocholate; the labeling of this polypeptide was less sensitive to the presence of taurocholate than the labeling of the other polypeptides and for a clear reduction in the extent of labeling, significant higher concentrations of taurocholate were necessary. In order to determine whether the hitherto unidentified bile salt-binding polypeptide of molecular weight 24,000 may be a further component of bile salt transport systems of the hepatocyte sinusoidal membrane, subfractions enriched with sinusoidal surfaces (4) were submitted to photoaffinity labeling with (3,3-azo-7α,12α-di-hydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid. The bile salt carrier protein of molecular weight 48,000 and membrane-bound albumin (M_r 67,000) were the predominantly labeled polypeptides whereas the polypeptide of molecular weight 54,000 was only slightly labeled; no significant labeling of the polypeptides having molecular weights of 33,000 and 24,000 occurred. Therefore, from these experiments it is unlikely that the hitherto unidentified bile salt-binding polypeptide of molecular weight 24,000 is a component of the hepatocyte sinusoidal membrane.

The labeling experiments presented here clearly demonstrate that for photoaffinity labeling studies a set of different photolabile derivatives with the photolabile group attached at different positions of the molecule is necessary. Only the use of such a set of photoprobes minimizes the possibility that a bile salt-binding protein will escape detection. The labeling of hepatocytes has been chosen as an example for such differences in the labeling patterns. Similar effects on the labeling pattern between 3,3-azo- and 7,7-azo-derivatives of taurocholate were found in labeling experiments of human serum, brush border membrane vesicles from rat small intestine and kidney (34), and basolateral membranes from rat renal cortex (35,36). In conclusion, the carbene-generating 3-diazirine-derivatives of bile salts described here are useful new photoprobes for the identification of bile salt binding proteins.

The authors thank Prof. Dr. E. Petzinger for his help in the experiments with isolated hepatocytes.

Manuscript received 8 March 1988, in revised form 27 October 1988, and in re-revised form 9 February 1989.

REFERENCES

1. Kramer, W., and Kurz, G. 1983. Photolabile derivatives of bile salts. Synthesis and suitability for photoaffinity labeling. *J. Lipid Res.* **24:** 910–923.
2. Kramer, W., H-P. Buscher, W. Gerok, and G. Kurz. 1979. Bile salt binding to serum components. Taurocholate incorporation into high-density lipoproteins revealed by photoaffinity labeling. *Eur. J. Biochem.* **102:** 1–9.
3. Kramer, W., U. Bickel, H-P. Buscher, W. Gerok, and G. Kurz. 1980. Binding proteins for bile acids in membranes of hepatocytes revealed by photoaffinity labeling. *Hoppe-Seyler's Z. Physiol. Chem.* **361:** 1307.
4. Kramer, W., U. Bickel, H-P. Buscher, W. Gerok, and G. Kurz. 1982. Bile salt binding polypeptides in plasma membranes of hepatocytes revealed by photoaffinity labeling. *Eur. J. Biochem.* **129:** 13–24.
5. Abberger, H., U. Bickel, H-P. Buscher, K. Fuchte, W. Gerok, W. Kramer, and G. Kurz. 1981. Transport of bile acids: lipoproteins, membrane polypeptides, and cytosolic proteins as carriers. *In* Bile Acids and Lipids. G. Paumgartner, A. Stiehl, and W. Gerok, editors. MTP Press, Lancaster, England. 233–246.
6. Wieland, T., M. Nassal, W. Kramer, G. Fricker, U. Bickel, and G. Kurz. 1984. Identity of hepatic membrane transport systems for bile salts, phalloidin, and antamanide. *Proc. Natl. Acad. Sci. USA.* **81:** 5232–5236.
7. von Dippe, P., and D. Levy. 1983. Characterization of the bile acid transport system in normal and transformed hepatocytes. Photoaffinity labeling of the taurocholate carrier protein. *J. Biol. Chem.* **258:** 8896–8901.
8. Burckhardt, G., W. Kramer, G. Kurz, and F. A. Wilson. 1983. Inhibition of bile salt transport in brush-border membrane vesicles from rat small intestine by photoaffinity labeling. *J. Biol. Chem.* **258:** 3618–3622.
9. Kramer, W., G. Burckhardt, F. A. Wilson, and G. Kurz. 1983. Bile-salt binding polypeptides in brush-border membrane vesicles from rat small intestine revealed by photoaffinity labeling. *J. Biol. Chem.* **258:** 3623–3627.
10. Burckhardt, G., W. Kramer, G. Kurz, and F. A. Wilson. 1987. Photoaffinity labeling studies of the rat renal sodium/bile salt cotransport system. *Biochem. Biophys. Res. Commun.* **143:** 1018–1023.
11. Abberger, H., H-P. Buscher, K. Fuchte, W. Gerok, U. Giese, W. Kramer, G. Kurz, and U. Zanger. 1983. Compartmentation of bile salt biosynthesis and transport revealed by photoaffinity labeling of isolated hepatocytes. *In* Bile Acids and Cholesterol in Health and Disease. G. Paumgartner, A. Stiehl, and W. Gerok, editors. MTP Press, Lancaster, England. 77–87.

12. Fricker, G., S. Schneider, W. Gerok, and G. Kurz. 1987. Identification of different transport systems for bile salts in sinusoidal and canalicular membranes of hepatocytes. *Biol. Chem. Hoppe-Seyler.* **368:** 1143–1150.
13. Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell. Biol.* **43:** 506–520.
14. Anwer, M. S., R. Kroker, and D. Hegner. 1976. Cholic acid uptake into isolated rat hepatocytes. *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 1477–1486.
15. Lamers, J. M. J., and W. C. Hülsmann. 1975. Inhibition of pyruvate transport by fatty acids in isolated cells from rat small intestine. *Biochim. Biophys. Acta.* **394:** 31–45.
16. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56:** 335–341.
17. Ruzicka, l., P. A. Plattner, and A. Heusser. 1943. 19. Steroide und Sexualhormone (91. Mitteilung). Über β'-|3α,7α,12β-Trioxy-nor-cholanoyl-(23)|-α',β'-butenolid), ein Homologes der digitaloiden Aglucone. *Helv. Chim. Acta.* **27:** 186–194.
18. Still, W. C., M. Kahn, and A. Mitra. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **43:** 2923–2925.
19. Norman, A. 1955. Preparation of conjugated bile acids using mixed carboxylic acid anhydrides. Bile acids and steroids. 34. *Ark. Kemi.* **8:** 331–342.
20. Jones, A. S., M. Webb, and F. Smith. 1949. Basic derivatives of steroids. 3-Amino-7:12-dihydroxy- and 3-amino-12-hydroxy-cholanic acid. *J. Chem. Soc.* **1949:** 2164–2700.
21. Tserng, K-Y. 1978. A convenient synthesis of 3-keto-bile acids by selective oxidation of bile acids with silver carbonate-Celite. *J. Lipid Res.*. **19:** 501–504.
22. Fetizon, M., and M. Golfier. 1968. Oxydation selective des alcohols par le carbonate d'argent. *C. R. Acad. Sci. Paris.* **267:** 900–903.
23. Lepage, G. A., A. Fontaine, and C. C. Roy. 1978. Vulnerability of keto bile acids to alkaline hydrolysis. *J. Lipid Res.* **19:** 505–509.
24. Leppick, R. A. 1983. Improved synthesis of 3-keto, 4-ene-3-keto, and 4,6-diene-3-keto bile acids.*Steroids.* **41:** 475–484.
25. Church, R. F. R., A. S. Kende, and M. J. Weiss. 1970. Diazirines. II. Synthesis and properties of small functionalized diazirine molecules. Some observations on the reaction of a diaziridine with the iodine-iodide ion system. *J. Org. Chem.* **35:** 2465–2471.
26. Church, R. F. R., and M. J. Weiss. 1965. Diazirines. I. Some observations on the scope of the ammonia-hydroxylamine-O-sulfonic acid diaziridine synthesis. The preparation of certain steroid diaziridines and diazirines. *J. Org. Chem.* **35:** 2465–2471.
27. Mosbach, E. H., W. Meyer, and F. E. Kendall. 1954. Preparation and $NaBH_4$ reduction of 7-ketocholanic acid. *J. Am. Chem. Soc.* **76:** 5799–5800.
28. Iida, T., and F. C. Chang. 1982. Potential bile acid metabolites. 6. Stereoisomeric 3,7-dihydroxy-5β-cholanic acids. *J. Org. Chem.* **47:** 2966–2972.
29. Kramer, W. 1981. Die Identifizierung gallensäurebindender Polypeptide durch Photoaffinitätsmarkierung. Synthese und Anwendung photolabiler Derivate der Gallensäuren. Inaugural Dissertation, Universität Freiburg. 1–316.
30. Henderson, C. J., and I. W. Percy-Robb. 1984. Synthesis and characterization of an iodinated bile-salt derivative for photoaffinity labeling. *Biochim. Biophys. Acta.* **795:** 257–264.
31. Fricker, G., G. Hugentobler, P. J. Meier, G. Kurz, and J. L. Boyer. 1987. Identification of a single sinusoidal bile salt uptake system in skate liver. *Am. J. Physiol.* **253:** G816–G822.
32. Ananthanarayanan, M., P. van Dippe, and D. Levy. 1988. Identification of the hepatocyte Na^{+}-dependent bile acid transport protein using monoclonal antibodies. *J. Biol. Chem.* **263:** 8338–8343.
33. Ruetz, S., G. Fricker, G. Hugentobler, K. Winterhalter, G. Kurz, and P. J. Meier. 1987. Isolation and characterization of the putative canalicular bile salt transport system of rat liver. *J. Biol. Chem.* **262:** 11324–11330.
34. Burckhardt, G., W. Kramer, F. A. Wilson, and G. Kurz. 1984. Characterization of the renal bile salt transport system by photoaffinity labeling. *Pflüger's Arch.* **400:** R 29.
35. Kramer, W., and G. Burckhardt. 1988. Identification of binding proteins for bile salts, β-lactam antibiotics and PAH in rat renal basolateral membranes by photoaffinity labeling. *Pflüger's Arch.* **411:** R 103.
36. Burckhardt, G., W. Kramer, and F. A. Wilson. 1989. Comparison of bile salt-binding proteins in basolateral membranes from small intestine, kidney and liver. *In* Hepatic Transport of Organic Substances. E. Petzinger, R. K-H. Kinne, and H. Sies, editors. Springer-Verlag, Berlin, Heidelberg. 297–298.