Fluorescent derivatives of bile salts. II. Suitability of NBD-amino derivatives of bile salts for the study of biological transport

U. Schramm,* A. Dietrich,* S. Schneider,[1,*] H-P. Buscher,† W. Gerok,† and G. Kurz[2,*]

Institut für Organische Chemie und Biochemie der Universität Freiburg,* D-7800 Freiburg, and Medizinische Universitätsklinik Freiburg,† D-7800 Freiburg, Germany

Abstract Interaction of unconjugated and taurine-conjugated NBD-amino-dihydroxy-5β-cholan-24-oic acids bearing the fluorophor in the 3α, 3β, 7α, 7β, 12α, or 12β position with albumin results in a small hypsochromic shift of the emission maximum and an increase in quantum yield, suggesting binding by hydrophobic interactions. The different unconjugated fluorescent bile salt derivatives are metabolized by intact rat liver in different ways. The unconjugated 3β-NBD-amino derivative is completely transformed to its taurine conjugate and secreted as such, whereas the 3α-NBD-amino derivative is completely transformed to a polar fluorescent compound not identical with its taurine conjugate. The unconjugated 7α- and 7β-NBD-amino derivatives are only partially conjugated with taurine and mainly secreted in unmetabolized form. The unconjugated 12α- and 12β-NBD-amino derivatives are not at all transformed to their taurine conjugates, but are partially metabolized to unidentified compounds. They are predominantly secreted as the unmetabolized compounds. In contrast to the unconjugated derivatives, all taurine-conjugated fluorescent bile salt derivatives are secreted into bile unmetabolized. With the exception of the 3α-compound, all synthesized taurine-conjugated fluorescent derivatives interfere with the secretion of cholyltaurine. Differential photoaffinity labeling studies using (7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-[2'-^{3}H(N)]aminoethanesulfonate as a photolabile derivative revealed that in liver cells all fluorescent bile salt derivatives interact with the same polypeptides as the physiological bile salts. The hepatobiliary transport of taurine-conjugated NBD-amino bile salt derivatives is, due to hydrophobic interactions, accompanied by an increase in fluorescence intensity which is favorable for the study of biological bile salt transport by fluorescence microscopy.—**Schramm, U., A. Dietrich, S. Schneider, H-P. Buscher, W. Gerok, and G. Kurz.** Fluorescent derivatives of bile salts. II. Suitability of NBD-amino derivatives of bile salts for the study of biological transport. *J. Lipid Res.* 1991. **32:** 1769–1779.

Supplementary key words bile salt-binding polypeptides • isolated hepatocytes • hepatobiliary transport • fluorescence microscopy • 4-nitrobenzo-2-oxa-1,3-diazol derivatives • photoaffinity labeling

Transport of bile salts in liver is essential for maintaining their effective enterohepatic circulation, thereby keeping their de novo biosynthesis at a low level. In the course of hepatic transcellular transport from blood to bile, conjugated bile salts are not metabolized, whereas unconjugated bile salts, the products of deconjugation by intestinal microorganisms, are subjected to reconjugation. At the cellular level, vectorial transport occurs from sinusoidal to canalicular membrane. In the intestine, the other organ participating in enterohepatic circulation, recycling of bile salts is ensured by reabsorption from the lumen and transport to blood. Thus, in contrast to hepatocytes, vectorial cellular transport occurs in the opposite direction from luminal to basal surface. In the kidney, bile salts are selectively reabsorbed from the glomerular filtrate and returned to the circulation. As in the intestine, cellular transport is directed from luminal to basal surface. In all three organs, which are highly complex with regard to their morphology and their functions, only distinct cells have the ability to transport bile salts.

In order to visualize bile salt transport, unconjugated and taurine-conjugated fluorescent derivatives of bile salts were synthesized by introduction of an NBD-amino group as fluorophor in different diastereomeric positions of the steroid structure (1). With the aid of these fluorescent derivatives of bile salts it should be possible to gain insights into the functional organization of bile salt transport at the organ level and at the cellular level by fluorescent microscopic studies. The NBD-amino group is a relatively small fluorophor with favorable optical properties (1). Its introduction results in structural changes of the

Abbreviations: 7,7-ACT, (7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-[2'-^{3}H(N)]aminoethanesulfonate; HPTLC, high performance thin-layer chromatography; LSC, liquid scintillation counting; NBD, 4-nitrobenzo-2-oxa-1,3-diazol; NBD-NC, N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-amino-dihydroxy-5β-cholan-24-oic acid; NBD-NCT, {N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-amino-dihydroxy-5β-cholan-24-oyl}-2'-aminoethanesulfonate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

[1]Present address: Abbott GmbH, Max-Planck-Ring 2, D-6200 Wiesbaden-Delkenheim, Germany.

[2]To whom correspondence should be addressed.

bile salt molecules, which may lead to alterations in their physiological behavior. To examine the suitability of the different unconjugated and taurine-conjugated fluorescent bile salt derivatives for the study of bile salt transport by fluorescence microscopy, their interaction with bile salt-binding proteins and their behavior in hepatic metabolism in the course of their transport from blood to bile was investigated. Preliminary accounts of the application of two of the derivatives for fluorescent microscopic studies have been presented (2-6).

MATERIALS AND METHODS

Materials

The NBD-amino derivatives of bile salts, cholyl[2'-^{3}H(N)]taurine, and 7,7-ACT (750-1500 GBq/mmol) were synthesized as described (1, 7). Silica gel plates (Kieselgel 60 without fluorescence indicator) for HPTLC were purchased from Merck (Merck, Darmstadt, Germany).

Animals

Male Wistar rats (Tierzuchtanstalt Jautz, Hannover, Germany) weighing 150-200 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.

Liver infusion experiments

An unconjugated fluorescent bile salt derivative (0.05 to 1.5 μmol) or 0.1 nmol of a tritium-labeled conjugated compound, dissolved in 0.5 ml of 0.15 M NaCl, was injected into a peripheral mesenterial vein of rats anesthetized with pentobarbital (3 mg of sodium pentobarbital/100 g body weight, i.p.). Assuming a portal blood flow of about 10 ml/min (8), the compounds were infused over a 1-min period so that their final concentration in blood did not exceed 150 μM. Bile was collected at different times beginning 10 min before the start of the injection. The nature of the secreted compounds was determined by TLC.

Constant infusion of cholyltaurine with tracer doses of [^{3}H]cholyltaurine was performed as described (9). The rate of infusion was chosen to approximate the known maximal rate of cholyltaurine excretion into bile (9). Twenty min after the onset of infusion, animals also received a bolus of 0.1 μmol of a conjugated fluorescent bile salt derivative.

Fluorescence microscopy

One mg of {N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oyl}-2'-aminoethanesulfonate, dissolved in 500 μl 0.15 M NaCl was injected into a peripheral mesenterial vein of rats as described above. Subsequently, liver biopsy from different lobes was performed at different times after start of injection. The specimens were shock-frozen in Freon 22 (Du Pont de Nemours, Bad Homburg, Germany) and stored in liquid nitrogen. Frozen sections, 5 μm thick, were prepared at -20°C with a cryotome Frigocut 2700 (Reichert-Jung, Nussloch, Germany). They were air-dried without fixation and immediately examined by incident light fluorescence using a fluorescence microscope Polyvar (Reichert-Jung) equipped with an excitation filter 450-495 nm, dichroic mirror 510 nm, and barrier filter 520-560 nm. Photographs were recorded with times of exposure of 4-30 sec on Kodak Ektachrome 400 film.

Isolation of hepatocytes

The standard isolation and incubation medium in all experiments with hepatocytes contained 118 mM NaCl, 4.74 mM KCl, 1.2 mM $MgCl_2$, 0.59 mM KH_2PO_4, 0.59 mM Na_2HPO_4, 24 mM $NaHCO_3$, 1.25 mM $CaCl_2$, and 5.5 mM D-glucose, and was saturated with carbogen (95% O_2/5% CO_2) and adjusted to pH 7.4. Isolated hepatocytes from livers of pentobarbital-anesthetized rats were prepared by collagenase perfusion (10). The yield of hepatocytes was about 2×10^8 cells/liver. The freshly isolated hepatocytes were allowed to recover by gently shaking the cell suspension ($2-3 \times 10^6$ cells/ml; 4-6 mg protein/ml) at 37°C under an atmosphere of carbogen for 20 min. Cell viability was estimated by determining Trypan blue exclusion and leakage of lactate dehydrogenase. Only cell suspensions with a viability of >90% were used. Experiments with freshly isolated hepatocytes were performed within 2 h after isolation.

Isolation of membrane subfractions

Plasma membrane subfractions enriched either with sinusoidal surfaces or predominantly composed of canalicular membranes were prepared from rat liver as described (11, 12).

Photoaffinity labeling and identification of labeled polypeptides

To 0.5 ml of a suspension of isolated hepatocytes ($2-3 \times 10^6$ cells/ml; 2-3 mg protein) in incubation medium or a suspension of membrane subfractions (1 mg protein) in phosphate buffer (150 mM, pH 7.4), 370 kBq of the photolabile bile salt derivative was added, and this was immediately transferred into the photolysis apparatus. Differential photoaffinity labeling experiments were performed in the presence of different concentrations (50-200 μM) of the fluorescent bile salt derivatives.

Photolysis and photoaffinity labeling were carried out at 30°C in a Rayonet RPR 100 reactor (The Southern Ultraviolet Company, Hamden, CT) equipped with 16 RPR 3500 Å lamps (7).

Discontinuous SDS-PAGE using vertical slab gels (200 × 180 × 2.8 mm) was performed as described (13).

Detection of radioactivity

Radioactivity in aqueous solutions and in bile was determined after addition of 4 ml of Quickszint 1 (Zinsser Analytic GmbH, Frankfurt, Germany) by liquid scintillation counting (RackBeta 1217, Pharmacia LKB, Freiburg, Germany). Radioactivity on thin-layer plates was detected with a radioscanner (Linear Analyser, Berthold, Wildbad, Germany). Detection of radioactivity in polyacrylamide gels was performed as described (14).

Separation of bile salt derivatives in bile

The fluorescent bile salt derivatives were detected on HPTLC plates using the solvent systems ethyl acetate–cyclohexane–acetic acid 23:7:3 (v/v/v) for unconjugated bile salt derivatives and n-butanol–acetic acid–water 9:2:1 (v/v/v) for conjugated bile salt derivatives.

Absorption and emission spectra

Ultraviolet absorption spectra were measured with a Perkin-Elmer UV/VIS-Spectrophotometer Lambda 5 (Perkin-Elmer GmbH, Überlingen, Germany). Fluorescence spectra were recorded with a fluorescence spectrophotometer connected with a data unit DC-SU-2 (Perkin-Elmer GmbH). Quantum yields were determined by using the quantum counter Basic Blue 3[2,7-bis-(diethylamino)-phenazoxonium-chloride].

RESULTS

Spectroscopic properties of the NBD-amino derivatives of bile salts in presence of albumin

The fluorescence of NBD-amino derivatives of bile salts is dependent on the hydrophobicity of their environment (1). Therefore, the influence of albumin, the main transport protein for bile salts in serum (15, 16), on the optical properties of taurine-conjugated NBD-amino-dihydroxy-cholanoic acids was investigated to determine the effects of protein binding. The presence of albumin causes a small hypsochromic shift of the longest wavelength absorption maxima, as shown for 7β-NBD-NCT, where the maximum is shifted from 497 nm in aqueous solution to 486 nm in presence of 5% albumin (**Fig. 1**). This hypsochromic shift is accompanied by a decrease of the extinction coefficient from ϵ = 25000 $M^{-1}cm^{-1}$ to ϵ = 21300 $M^{-1}cm^{-1}$ at the absorption maximum and the disappearance of the shoulder beside the maximum. The influence of albumin on the longest wavelength absorption maxima and on the corresponding extinction coefficients was qualitatively similar for all conjugated fluorescent bile salt derivatives and showed only small quantitative differences. As to be expected, the emission maxima of the

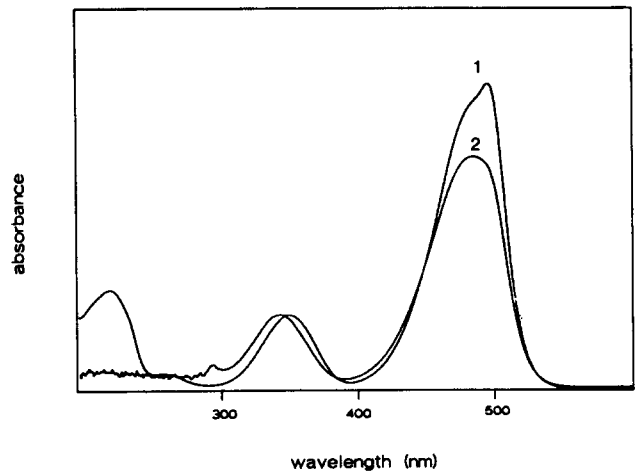

Fig. 1. Absorption spectra of 7β-NBD-NCT (5 μM). (1) Phosphate buffer, pH 7.4; (2) phosphate buffer, pH 7.4+albumin (5%).

fluorescent bile salt derivatives also exhibited a hypsochromic shift in the presence of albumin, as shown for 7β-NBD-NCT, where the maximum shifted from 567 nm to 550 nm (**Fig. 2, Table 1**). In addition there was a moderate increase in quantum yield from 0.025 to 0.04. In principle, the emission maxima and the quantum yields of the other taurine-conjugated fluorescent bile salt derivatives showed qualitatively similar changes in the presence of albumin (Table 1). Small differences in quantum yields are due to differences in position and orientation of the fluorophor interacting with the protein. Thus, relatively the highest increase in quantum yield can be observed with 3β-NBD-NCT, whereas with 7α-NBD-NCT only an insignificant increase in quantum yield was seen.

Metabolism and secretion of the unconjugated fluorescent bile salt derivatives

Overall metabolism and transport of the unconjugated fluorescent bile salt derivatives in liver were studied, to evaluate their suitability as bile salt analogues, by determination of excretion rate into bile and qualitative analysis of excreted products. Infusion experiments, where each of the fluorescent compounds was applied as a bolus into a mesenterial vein, showed that all unconjugated fluorescent bile salt derivatives are taken up by the liver and secreted into bile either unmetabolized or transformed to metabolites with a secretion maximum of 5–6 min. Analysis of the collected bile samples by TLC revealed qualitatively that the different unconjugated fluorescent bile salt derivatives are metabolized differently, depending on the position of the fluorescent group.

The 3α-derivative appeared in bile completely metabolized as a fluorescent bile salt derivative that was more polar than the taurine-conjugated 3α-compound. The unconjugated 3β-compound was secreted into bile, with a

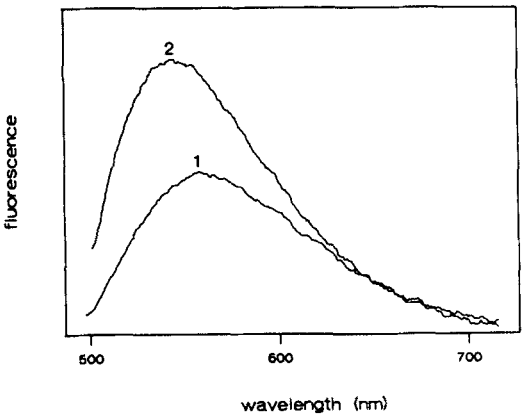

Fig. 2. Emission spectra of 7β-NBD-NCT (5 μM). (1) Phosphate buffer, pH 7.4; (2) phosphate buffer, pH 7.4+albumin (5%).

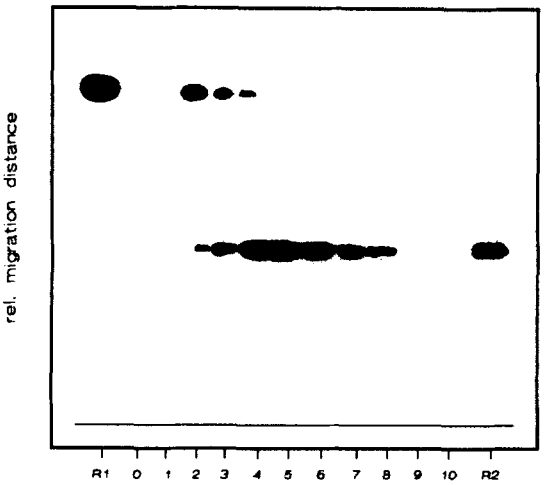

Fig. 3. TLC of bile samples collected after injection of a bolus of 3β-NBD-NC (1.5 μmol) into a rat mesenterial vein. Solvent system: n-butanol–acetic acid–water 9:2:1 (v/v/v). R1: 3β-NBD-NC; R2: 3β-NBD-NCT; 0: reference bile; 1–10: samples collected in 1-min intervals starting with injection of 3β-NBD-NC.

secretion maximum of about 5 min, comparable to cholate, almost completely as its taurine conjugate (**Fig. 3**). The unconjugated 7-isomers of the fluorescent bile salt derivatives were both secreted into bile mainly unmetabolized and only to about 15% conjugated with taurine; other biotransformation products could not be detected under the conditions used. The extent of conjugation was dependent on the concentration of the 7-isomers in blood or perfusing medium and became insignificant at low concentrations. Both 12-isomers appeared in bile predominantly unconjugated and about 15% metabolized to fluorescent compounds, which were less polar according to their chromatographic behavior. The rate of metabolization decreased with decreasing concentration of the 12-isomers in blood or perfusing medium.

Metabolism and secretion of the taurine-conjugated fluorescent bile salt derivatives

For studying the overall transport of the conjugated fluorescent bile salt derivatives in liver, infusion experiments were performed with each of the different diastereomers of the conjugated fluorescent bile salt derivatives. In order to easily detect possible metabolism of the NBD-amino function, which could cause vanishing of fluorescence, all infusion experiments were performed with the tritium-labeled compounds, with the label in the taurine moiety of the molecule. Each of the conjugated fluorescent bile salt derivatives was secreted with a secretion maximum comparable to that of cholyltaurine as demonstrated for 3β-NBD-NCT which showed maximal secretion at 5 ± 0.5 min (**Fig. 4**). All taurine-conjugated fluorescent bile salt derivatives were secreted into bile unmetabolized, and, with the exception of 3α-NBD-NCT, in all cases more than 95% of the applied compound could be detected in bile within 30 min (Fig. 4, insert). Out of all taurine-conjugated fluorescent derivatives only 3α-NBD-NCT exhibited a retarded secretion; after 30 min only 40% of this derivative appeared in bile (Fig. 4, insert). The secretion of the conjugated fluorescent bile salt derivatives over the path used by physiological bile salts could be demonstrated by inhibition of the biliary maximal secretion rate (T_m) of cholyltaurine in rat liver (**Fig. 5**). Infusion of cholyltaurine rapidly led to an in-

TABLE 1. Emission maxima and quantum yield of taurine-conjugated NBD-amino derivatives of bile salts (5 μM) in phosphate buffer, pH 7.4, and in phosphate buffer, pH 7.4 + albumin (5%)

	Emission Maximum (nm)		Quantum Yield	
Compound	Buffer, pH 7.4	Buffer, pH 7.4 + Albumin (5%)	Buffer, pH 7.4	Buffer, pH 7.4 + Albumin (5%)
3α-NBD-NCT	568	538	0.022	0.042
3β-NBD-NCT	568	536	0.021	0.103
7α-NBD-NCT	562	558	0.026	0.031
7β-NBD-NCT	567	550	0.025	0.041
12α-NBD-NCT	560	556	0.027	0.039
12β-NBD-NCT	569	561	0.036	0.051

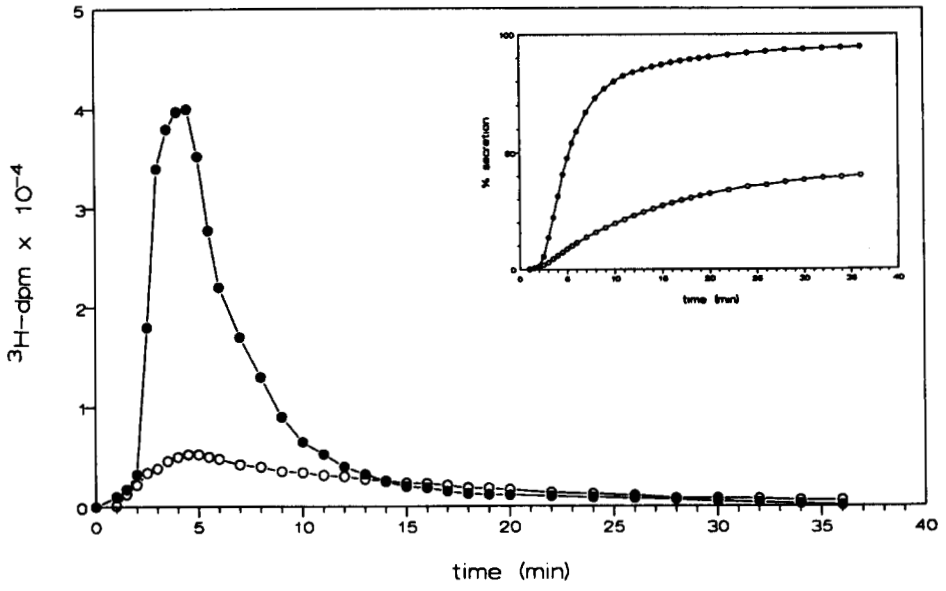

Fig. 4. Time dependency of biliary secretion of 3β-NBD-NCT (●) and 3α-NBD-NCT (○) after a bolus injection of 0.1 nmol of the respective tritium-labeled derivative into a rat mesenterial vein. Insert: cumulative % of dose injected.

crease in its biliary concentration and secretion rate, reaching a steady state after 20 min. Administration of small concentrations of 7β-NBD-NCT did not result in an increase of bile flow, as expected for saturation conditions, and there was a clear decrease of the secretion rate of cholyltaurine concomitant with the appearance of the fluorescent bile salt derivative in bile. After the fluorescent bile salt derivative had been secreted, secretion of cholyltaurine increased again reaching the original secretion rate after 50 min. The taurine-conjugated 3β- and the 7α-derivative as well as both 12-isomers behaved in just the same way. In contrast to all other taurine-conjugated fluorescent bile salt derivatives, 3α-NBD-NCT was excreted much slower (Fig. 4) and no influence on the biliary maximal excretion rate (T_m) of cholyltaurine in rat liver could be observed.

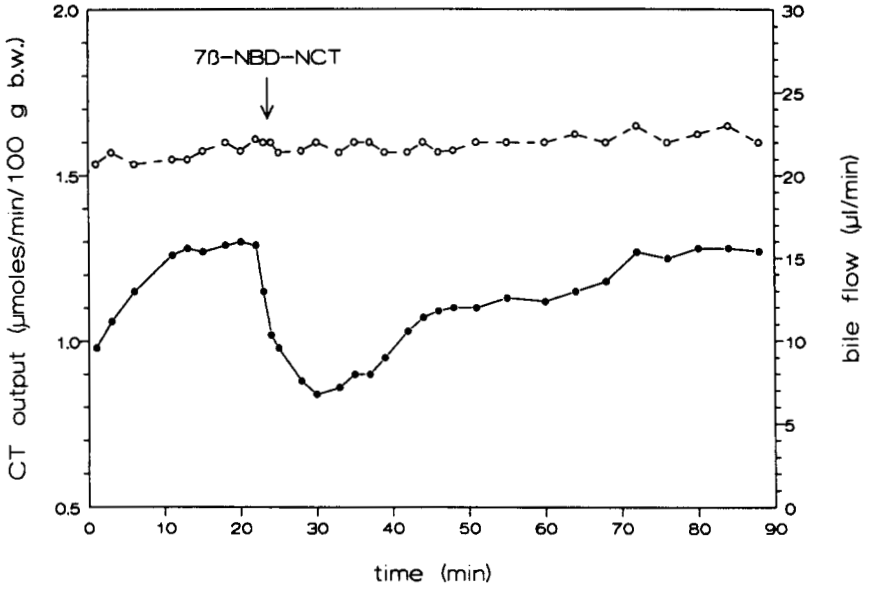

Fig. 5. Bile flow (○) and cholyltaurine secretion in bile (●) during a constant infusion of cholyltaurine (1.6 μmol/min/100 g b.w.). Injection of an additional bolus of 7β-NBD-NCT (0.1 μmol) at the time indicated by the arrow.

Interaction of the taurine-conjugated fluorescent bile salt derivatives with bile salt binding polypeptides in liver

In order to ascertain that the fluorescent bile salt derivatives interact with the same binding polypeptides as the physiologic bile salts, differential photoaffinity labeling studies using the photolabile bile salt derivative 7,7-ACT were performed in the presence of the conjugated fluorescent bile salt derivatives. Photoaffinity labeling using 7,7-ACT requires irradiation at 350 nm and absorption of light at this wavelength by the NBD-amino group of the fluorescent bile salt derivatives had to be taken into account. Absorption of light by the fluorescent derivatives does not change the shape of the absorption spectra of the photolabile bile salt derivative, but decreases the intensity of light at 350 nm used for photolysis. In order to consider the absorption of light by the fluorescent derivative during photolysis, control experiments were performed in which the test tube containing the photolabile derivative was enclosed by a solution of the fluorescent bile salt derivative having the same concentration and pathlength as those used for the differential photoaffinity labeling experiments. Photolysis of the 7,7-azo-derivative revealed a prolongation of the half-life time of the photolabile bile salt derivative from 2.3 min in the absence to 4.5 min in the presence of 200 μM of a fluorescent bile salt derivative, the highest concentration used. In order to guarantee photolysis of at least 95% of the photolabile bile salt derivative in the presence of all concentrations of a fluorescent derivative, photolyses times for the differential photoaffinity labeling experiments were fixed to 20 min.

The interaction of bile salts with albumin was established by photoaffinity labeling using 7,7-ACT (7). Photoaffinity labeling in the presence of each of the fluorescent bile salt derivatives resulted in a clear decrease of incorporation of radioactivity into albumin as compared with the control, confirming that the fluorescent bile salt derivatives interact with this extracellular transport protein (data not shown).

Photoaffinity labeling of plasma membrane subfractions enriched with sinusoidal membranes revealed bile salt-binding polypeptides with apparent molecular weights of 67,000, 54,000, 48,000, and 43,000 (11). The extent of labeling of all these polypeptides decreased in the presence of increasing concentrations of each of the fluorescent bile salt derivatives, as shown with 12α-NBD-NCT (**Fig. 6**). These results clearly indicate that the fluorescent bile salt derivatives interact with all the polypeptides that were identified in subfractions enriched with sinusoidal membranes as bile salt binding proteins.

Photoaffinity labeling of plasma membrane subfractions enriched with canalicular membranes with 7,7-ACT resulted in the predominant labeling of a canalicular membrane polypeptide with the apparent molecular weight of about 100,000, shown to be involved in the secretion of bile salts (2, 17–19). Differential photoaffinity labeling experiments using a membrane subfraction enriched with bile canalicular membranes with 7,7-ACT clearly showed a decrease of incorporation of radioactivity into this polypeptide in the presence of every applied fluorescent bile salt derivative, as demonstrated for 7α-NBD-NCT (**Fig. 7**). The decrease in labeling of the polypeptide with the apparent molecular weight of 100,000 proves that the fluorescent bile salt derivatives also interact at the canalicular membrane with the same polypeptide as physiological bile salts.

Photoaffinity labeling of isolated hepatocytes revealed, in addition to bile salt binding-membrane polypeptides, cytosolic polypeptides with apparent molecular weights of 54,000, 38,000, 32,000, and 14,000 (20, 21) (**Fig. 8**). Differential photoaffinity labeling experiments with isolated hepatocytes in the presence of each of the conjugated fluorescent bile salt derivatives showed not only a de-

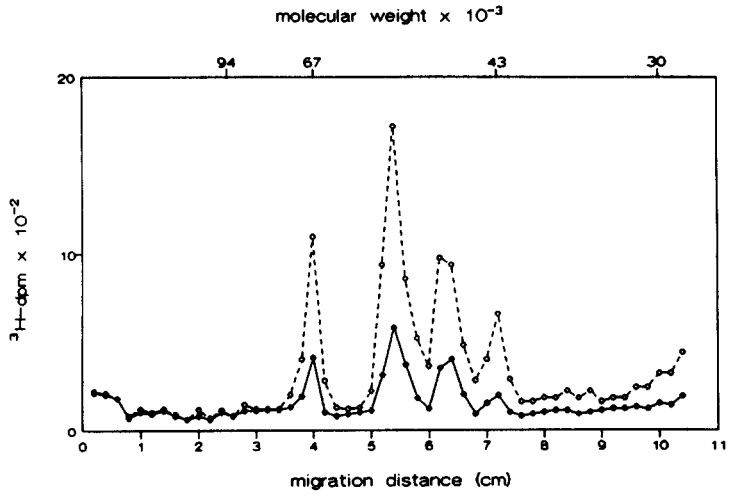

Fig. 6. Distribution of radioactivity after SDS-PAGE of a plasma membrane subfraction from rat liver enriched with sinusoidal membranes obtained after photoaffinity labeling with 7,7-ACT in the absence (○) and in the presence (●) of 100 μM 12α-NBD-NCT. Total acrylamide concentration of the gel was 10.5% at a ratio of acrylamide:bisacrylamide of 97.2:2.8.

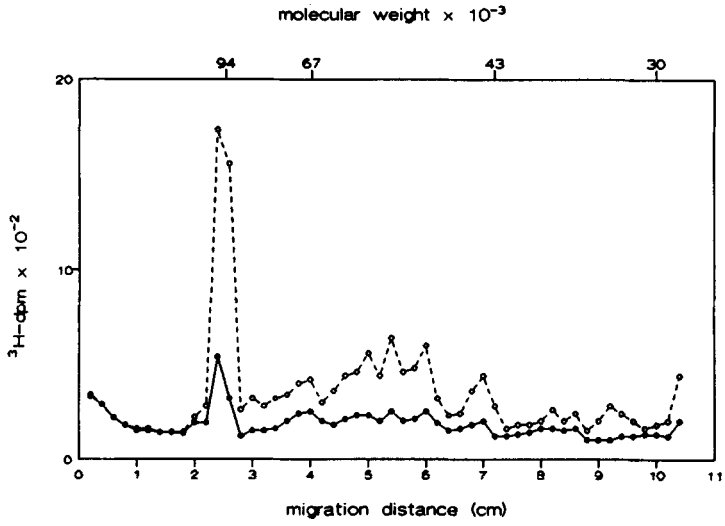

Fig. 7. Distribution of radioactivity after SDS-PAGE of a plasma membrane subfraction from rat liver enriched with canalicular membranes obtained after photoaffinity labeling with 7,7-ACT in the absence (○) and in the presence (●) of 100 μM 7α-NBD-NCT. Total acrylamide concentration of the gel was 10.5% at a ratio of acrylamide:bisacrylamide of 97.2:2.8.

creased labeling of all bile salt binding membrane polypeptides, but also a decrease of incorporation of radioactivity into all bile salt binding cytosolic polypeptides (Fig. 8).

Whereas in the presence of each of the conjugated fluorescent bile salt derivatives photoaffinity labeling of all bile salt-binding polypeptides of hepatocytes was decreased, the extent of the decrease was different for the different fluorescent derivatives and for the different polypeptides.

Visualization of transport in rat liver

In order to demonstrate the applicability of the taurine-conjugated NBD-amino bile salt derivatives for fluorescent microscopic studies, the appearance of fluorescence of 7β-NBD-NCT in rat liver is shown exemplarily by fluorescence microscopy of a frozen rat liver section (**Fig. 9a**). Three minutes after a bolus injection of 7β-NBD-NCT into a mesenterial vein, a yellow fluorescence was visible in the hepatocytes (Fig. 9a), and the sinusoids, which could be identified on the corresponding phase contrast micrograph, appeared dark (Fig. 9b). Intracellularly the nuclei stood out dark against the extranuclear fluorescence, which was not distributed evenly, but increased in the pericanalicular areas. The secretion of the fluorescent derivative into the bile canaliculi became beautifully visible in the upper right quadrant of the fluorescence micrograph (Fig. 9a), where a coherent system of canaliculi appeared fluorescent.

DISCUSSION

One of the major advantages of fluorescence spectroscopy is the environmental sensitivity of fluorescent probes. Bile salts are amphipathic compounds and as such they are bound, extracellularly as well as intracellularly to pro-

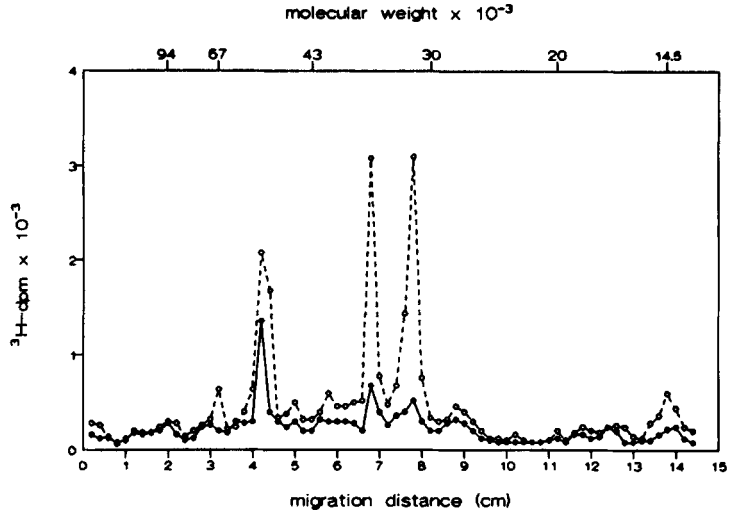

Fig. 8. Distribution of radioactivity after SDS-PAGE of cytosolic polypeptides obtained after photoaffinity labeling of isolated rat hepatocytes with 7,7-ACT in the absence (○) and in the presence (●) of 100 μM 3β-NBD-NCT. Cytosolic fraction was obtained by three times freezing and thawing the cell suspension and subsequent centrifugation of the homogenate at 175000 *g*. Total acrylamide concentration of the gel was 12% at a ratio of acrylamide:bisacrylamide of 97.2:2.8.

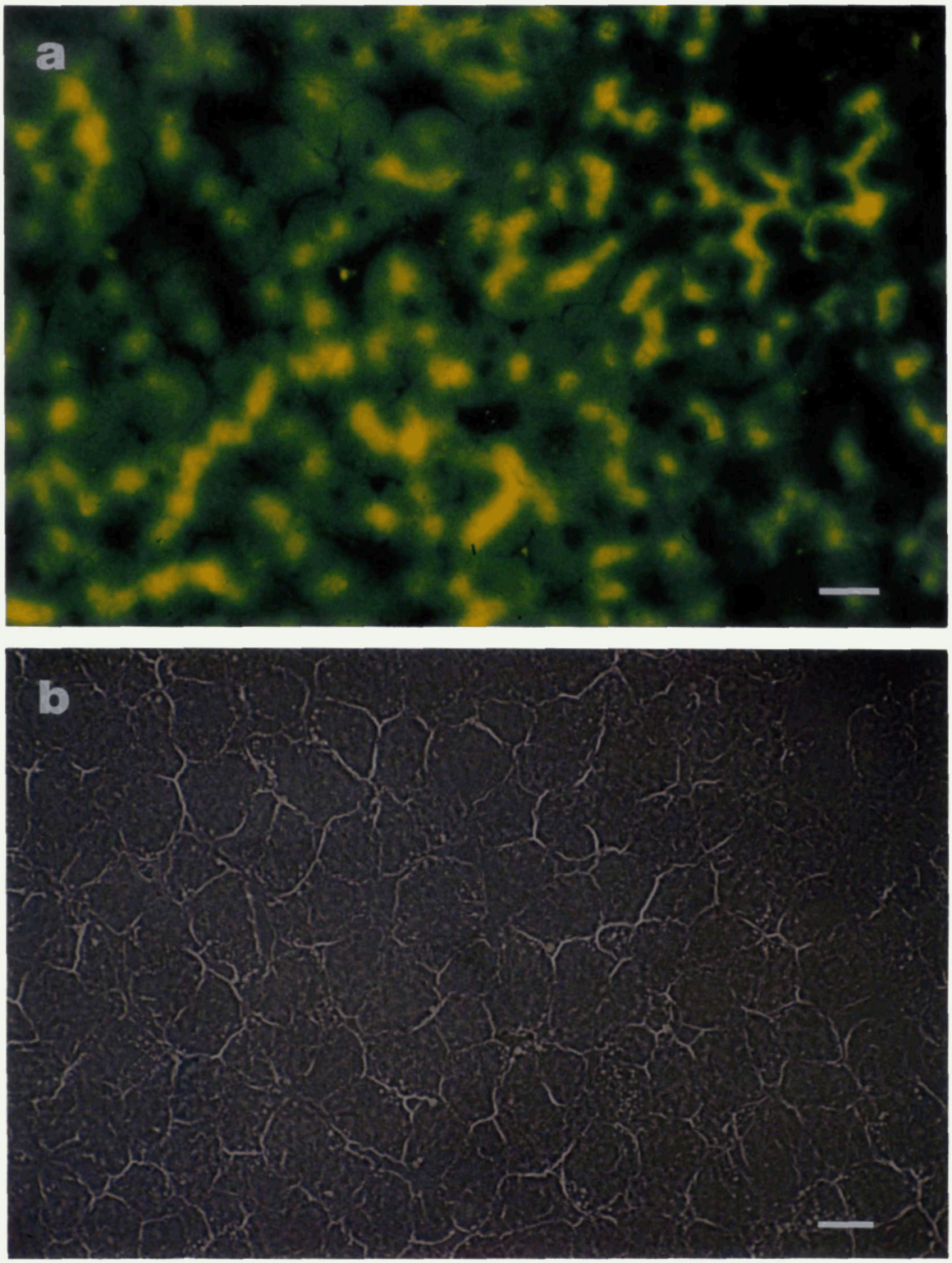

Fig. 9. Distribution of fluorescence in a frozen section (5 μm) of rat liver 3 min after injection of 0.5 mg 7β-NBD-NCT dissolved in 500 μl 0.15 M NaCl into a mesenterial vein. a: fluorescence micrograph; b: phase contrast micrograph; bar = 20 μm.

teins, presumably by the participation of hydrophobic interactions. Thus, with the binding of a fluorescent bile salt derivative to proteins, a shift of the emission maximum to smaller wavelengths and an increase in quantum yield is expected. The interaction of the fluorescent bile salt derivatives with albumin, the main transport protein for bile salts in serum, results in hypsochromic shifts of the emission maximum in addition to moderate increases in quantum yield (Table 1). These changes in emission properties resemble those that occur when the polarity of the solvent is reduced (1) and are compatible with the assumption of hydrophobic interactions between the protein and the bile salts. Because all taurine-conjugated fluorescent bile salt derivatives bind to albumin, as proved by differential photoaffinity labeling, the different changes in the emission properties, mainly the increases in quantum yield, indicate that the fluorophor, depending on its position in the bile salt molecule, interacts differently with hydrophobic domains of albumin. The NBD-amino group in the 3β-position, exhibiting a relatively high increase in quantum yield of about 5-fold, must interact more strongly with hydrophobic domains of albumin than other derivatives, which show only slight increases in quantum yield. In contrast, the NBD-amino group in the 7α-position, showing only an insignificant change in quantum yield, does not participate in hydrophobic interactions and may be mainly orientated to the aqueous phase. An increase in quantum yield due to hydrophobic interactions is favorable for the detection of the fluorescent bile salts in intact biological systems by fluorescence microscopy. The intensity of the fluorescence, shown for the hepatobiliary transport of 7β-NBD-NCT (Fig. 9a), opens new possibilities for the study of bile salt transport with the aid of fluorescent bile salt derivatives.

Unconjugated bile salts are taken up in liver by hepatocytes where they are intracellularly conjugated with taurine or glycine and subsequently secreted into bile. The total process comprises transport across membranes as well as the more specific enzymatic reactions involved in conjugation. In order to examine whether the physiological behavior of unconjugated bile salts is influenced by the introduction of the NBD-amino group, the metabolism of the unconjugated fluorescent derivatives had especially to be taken into consideration. All unconjugated fluorescent bile salt derivatives are metabolized by intact rat liver. However, dependent on the structure and the concentration of the fluorescent compound applied, the pathway and the extent of metabolism are different.

Metabolization of 3α-NBD-NC leads practically quantitatively to a compound that was more polar than the taurine conjugate according to its chromatographic behavior. The structure of the biotransformation product of 3α-NBD-NC has not yet been elucidated and could be the result of hydroxylation, glucuronidation, and/or sulfation, in order to facilitate biliary elimination. Under physiological conditions this biotransformation is not usual for bile salts and makes 3α-NBD-NC unusable for the study of bile salt transport and physiological metabolism of unconjugated bile salts, but might be of interest for the investigation of distinct biotransformation reactions. In contrast to the 3α-derivative, 3β-NBD-NC behaves like a physiological bile salt and appears in bile completely as its taurine conjugate (Fig. 3). Thus, the introduction of a relative bulky group in the axial 3β-position of bile salts does not impede conjugation and the 3β-NBD-amino derivative may therefore be suitable for studies relevant to hepatobiliary transport of unconjugated bile salts.

Introduction of the NBD-amino group in the 7-position, whether axial or equatorial, leads to a dependency of the extent of conjugation on the applied concentration of the unconjugated fluorescent derivatives. Whereas at low concentrations both derivatives, 7α- and 7β-NBD-NCT, are not metabolized, at higher concentrations taurine conjugation occurs to a considerable extent. It can be assumed that the NBD-amino substituent in the 7-position does not preclude conjugation but causes an increase of the K_m value of at least one of the enzymes involved in the conjugation process. Because only at low concentrations are the unconjugated fluorescent 7-NBD amino derivatives excreted unmetabolized, 7α-NBD-NC and 7β-NBD-NC may be used at these low concentrations for the study of hepatobiliary transport of unconjugatable bile salts by fluorescence microscopy.

The NBD-amino group in the 12-position, being closer to the carboxylate of the side chain, prevents taurine conjugation completely. Both 12-isomers, 12α-NBD-NC and 12β-NBD-NC, are secreted into bile predominantly unmetabolized and only when applied at higher concentrations are they partially metabolized. These metabolites, not further analyzed, were less polar than the unconjugated compounds according to their chromatographic behavior, possibly by transformation of one of the free hydroxyl groups to a less polar function. The rate of metabolism decreases with decreasing concentration of the 12-isomers in blood or perfusing medium and this may, as with the 7-derivatives, allow their application in low concentrations for fluorescent microscopic studies of hepatobiliary transport of unconjugatable bile salts.

Metabolic studies with the unconjugated fluorescent bile salt derivatives showed that the introduction of a residue, relatively small as fluorophor but relatively bulky for a bile salt, into different positions of the physiological molecule has marked discriminative effects on their intracellular transformation. Whereas the α- and the β-isomers of both the 7- and the 12-NBD-amino substituted unconjugated bile salts are excreted unmetabolized when applied at low concentrations and partly metabolized at higher concentrations, introduction of the fluorophor in the 3 position leads to a completely different behavior, depending on the orientation of the NBD-amino group. The

3α-NBD-amino derivative is biotransformed in a way not physiological for unconjugated bile salts, and only the 3β-NBD-amino derivative is almost completely secreted as its taurine conjugate. Thus, the 3β-NBD-amino derivative is, out of all the unconjugated fluorescent bile salt derivatives, of outstanding interest for the study of transport and metabolism of unconjugated bile salts by fluorescence microscopy.

The taurine-conjugated fluorescent bile salt derivatives must be transported through liver and secreted into bile just as the physiological conjugated bile salts without being metabolized if they are to be applicable for the visualization of bile salt transport by fluorescence microscopy.

With the exception of 3α-NBD-NCT, all taurine-conjugated fluorescent bile salt derivatives behave as physiological conjugated bile salts. They are completely secreted unmetabolized with about the same secretion maximum as cholyltaurine (Fig. 4). Biliary secretion of cholyltaurine is inhibited by each of the conjugated fluorescent derivatives likewise with the exception of the 3α-derivative (Fig. 5). Because secretion is the rate-limiting step in overall hepatobiliary transport of bile salts (9), these observations favor the hypothesis that cholyltaurine and these taurine-conjugated fluorescent bile salt derivatives share a common secretion pathway. Only the 3α-derivative makes an exception, exhibiting a retarded secretion and showing no inhibition of the maximal biliary secretion rate (T_m) of cholyltaurine in rat liver. This demonstrates that excretion of 3α-NBD-NCT into bile occurs by a transport system different from the bile salt-secreting system.

Concerning metabolism and secretion, all taurine-conjugated fluorescent derivatives, with the exception of 3α-NBD-NCT, are true analogues of cholyltaurine and should be suitable for the study of hepatobiliary transport of bile salts. However, depending on the position and orientation of the NBD-amino group, the taurine-conjugated fluorescent bile salt derivatives differ from each other in sinusoidal uptake velocity. A detailed kinetic study will be presented in a later publication.

In order to be relevant for the study of bile salt transport by fluorescence microscopy, there must be a guarantee that the fluorescent derivatives interact in all sections of transcellular transport with the same polypeptides as the physiological reference molecules. Interaction of bile salts with polypeptides, even with uncharacterized ones, may be favorably detected by photoaffinity labeling using appropriate photolabile derivatives (2, 4, 7, 12, 21, 22). Competition of ligands for binding sites of bile salts may be recognized by differential photoaffinity labeling experiments. Photoaffinity labeling of biological systems of different complexity using the photolabile bile salt derivative 7,7-ACT was performed in the presence of each of the conjugated fluorescent bile salt derivatives. With regard to the absorption of light of wavelength 350 nm, necessary for photoactivation of 7,7-ACT, by the NBD-amino group, the irradiation time was adequately prolonged.

The hepatobiliary transport of bile salts comprises their uptake from blood, where the bile salts are bound to albumin, into hepatocytes through the sinusoidal membrane, their intracellular translocation, and their secretion into bile through the canalicular membrane. In order to demonstrate that the fluorescent derivatives interact with the same binding polypeptides as physiological bile salts, differential photoaffinity labeling studies were performed with albumin to prove binding by the extracellular transport protein for bile salts, with different plasma membrane subfractions of liver to show the interaction with membrane polypeptides, and with isolated hepatocytes to identify, in addition, the interactions of the fluorescent bile salt derivatives with the intracellular proteins.

All differential photoaffinity labeling studies revealed a clear decrease of labeling of all polypeptides interacting with bile salts as compared with the controls in absence of the fluorescent bile salt derivatives (Figs. 6–8). The extent of the decrease was different for the different fluorescent derivatives and for the different polypeptides. This shows, as expected, that the different positions of the NBD-amino group in the fluorescent bile salt derivatives have an influence on the interaction with the distinct polypeptides. The results of the differential photoaffinity labeling experiments clearly show that all fluorescent bile salt derivatives, even the 3α-NBD-NCT, interact in isolated hepatocytes and in membrane subfractions enriched either with sinusoidal or canalicular membranes with the same polypeptides as the physiological bile salts.

Hepatobiliary overall transport and differential photoaffinity labeling studies indicate that, with the exception of 3α-NBD-NCT, the taurine-conjugated fluorescent bile salt derivatives are handled in liver as conjugated bile salts. However, it must be taken into account that the introduction of the relatively bulky NBD-group must lead to quantitative changes of distinct properties of the physiological bile salt. The extent of these quantitative changes depends on the different positions of the NBD-amino group at the steroid structure and will influence transport kinetics if more than one transport system is operative as in hepatic uptake (5, 22–24). Preliminary uptake studies using freshly isolated hepatocytes show a mutual competitive inhibition of uptake between 7β-NBD-NCT and cholyltaurine (5).

The chemical and optical properties of the NBD-amino derivatives of bile salts make them useful tools for fluorescent microscopic studies. Depending on the biological problem to be investigated, the suitable fluorescent bile salt derivative, either unconjugated or conjugated, must be selected. For the study of bile salt transport by fluorescence microscopy, different taurine-conjugated diastereomeric fluorescent derivatives are available. According to the specificity of transport, which may be different in cells

of different bile salt transporting organs, the appropriate fluorescent derivative must be experimentally predetermined and then chosen. Only under such precautions can fluorescence microscopy be applied to disclose the functional state of bile salt transport under physiological and pathological conditions.

The authors express their gratitude to Dipl. Chem. W. Pachali and Prof. Dr. J. Heinze from the Institut für Physikalische Chemie der Universität Freiburg for the fluorescence spectra. This investigation was supported by the Deutsche Forschungsgemeinschaft and the Fritz-Thyssen-Stiftung. One of us (U. Schramm) is indebted to the Böhringer-Ingelheim-Fonds for a scholarship.

Manuscript received 25 March 1991 and in revised form 16 August 1991.

REFERENCES

1. Schneider, S., U. Schramm, A. Schreyer, H-P. Buscher, W. Gerok, and G. Kurz. 1991. Fluorescent derivatives of bile salts. I. Synthesis and properties of NBD-amino derivatives of bile salts. *J. Lipid Res.* **32:** 1755–1767.
2. 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.
3. Buscher, H-P., W. Gerok, G. Kurz, and S. Schneider. 1985. Visualization of bile salt transport with fluorescent derivatives. *In* Enterohepatic Circulation of Bile Acids and Sterol Metabolism. G. Paumgartner, A. Stiehl and W. Gerok, editors. MTP-Press, Lancaster, England. 243–247.
4. Buscher, H-P., W. Gerok, M. Köllinger, G. Kurz, M. Müller, A. Nolte, and S. Schneider. 1988. Transport systems for amphipathic compounds in normal and neoplastic hepatocytes. *Adv. Enzyme Regul.* **27:** 173–192.
5. Buscher, H-P., G. Fricker, W. Gerok, G. Kurz, M. Müller, S. Schneider, U. Schramm, and A. Schreyer. 1987. Hepatic transport systems for bile salts: localization and specificity. *In* Bile Acids and the Liver with an Update on Gallstone Disease. G. Paumgartner, A. Stiehl and W. Gerok, editors. MTP-Press, Lancaster, England. 95–110.
6. Buscher, H-P., W. Gerok, G. Kurz, U. Schramm, and H. Thom. 1988. Hepatocyte primary culture and bile salt transport. *In* Trends in Bile Acid Research. G. Paumgartner, A. Stiehl and W. Gerok, editors. MTP-Press, Lancaster, England. 133–142.
7. Kramer, W., and G. Kurz. 1983. Photolabile derivatives of bile salts. Synthesis and suitability for photoaffinity labeling. *J. Lipid Res.* **24:** 910–923.
8. Ross, G., F. N. White, A. W. Brown, and A. Kolin. 1966. Regional blood flow in the rat. *J. Appl. Physiol.* **21:** 1273–1275.
9. Paumgartner, G., K. Sauter, H. P. Schwarz, and R. Herz. 1973. Hepatic excretory transport maximum for free and conjugated cholate in the rat. *In* The Liver. Quantitative Aspects of Structure and Function. Karger, Basel, Switzerland. 337–344.
10. 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.
11. 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 labelling. *Eur. J. Biochem.* **129:** 13–24.
12. Meier, P. J., E. Z. Sztul, A. Reuben, and J. L. Boyer. 1984. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. *J. Cell Biol.* **98:** 991–1000.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **277:** 680–685.
14. Falk, E., M. Müller, M. Huber, D. Keppler, and G. Kurz. 1989. Direct photoaffinity labeling of leukotriene binding sites. *Eur. J. Biochem.* **186:** 741–747.
15. Burke, C. W., B. Lewis, B. L. D. Pauveliwalla, and S. Tabaqchali. 1971. The binding of cholic acid and its taurine conjugate to serum proteins. *Clin. Chim. Acta.* **32:** 207–214.
16. Kakis, G., I. M. Yousef, and M. M. Fischer. 1977. Studies on the binding of bile acids by plasma proteins. *Gastroenterology.* **72:** 1178.
17. 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.
18. Ruetz, S., G. Hugentobler, and P. J. Meier. 1988. Functional reconstitution of the canalicular bile salt transport system of rat liver. *Proc. Natl. Acad. Sci. USA.* **85:** 6147–6157.
19. Sippel, S. J., M. Ananthanarayanan, and F. J. Suchy. 1990. Isolation and characterization of the canalicular membrane bile acid transport protein of rat liver. *Am. J. Physiol.* **258:** G728–G737.
20. 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.
21. Kurz, G., M. Müller, U. Schramm, and W. Gerok. 1989. Identification and function of bile salt binding polypeptides of hepatocyte membrane. *In* Hepatic Transport of Organic Substances. E. Petzinger, R. K-H. Kinne and H. Sies, editors. Springer-Verlag, Berlin, Heidelberg. 267–278.
22. Frimmer, M., and K. Ziegler. 1988. The transport of bile acids in liver cells. *Biochim. Biophys. Acta.* **947:** 75–99.
23. Berk, P. D., B. J. Potter, and W. Stremmel. 1987. Role of the plasma membrane binding proteins in the hepatocellular uptake of albumin-bound organic anions. *Hepatology.* **7:** 165–176.
24. Tiribelli, C., S. Bellentani, G. C. Lunazzi, and G. Sottocasa. 1989. Role and nature of plasma membrane carrier proteins in the hepatic transport of organic anions. *J. Gastroenterol. Hepatol.* **4:** 195–205.