Functional significance of interaction of H-FABP with sulfated and nonsulfated taurine-conjugated bile salts in rat liver

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Abstract To identify the cytosolic proteins of rat hepatocytes involved in transcellular transport of sulfated and taurine-conjugated bile salts in comparison with only taurine-conjugated bile salts, photoaffinity labeling studies were performed with [^{3}H]-7,7-ASLCT (7,7-azi-3α-sulfatolithocholyl[2′-^{3}H(N)]taurine), [^{3}H]-7,7-ACT ((7,7-azi-3α,12α-dihydroxy-5β-cholan-24-oyl)-2′-[2′-^{3}H(N)]aminoethanesulfonate), and [^{3}H]-7,7-ALCT (7,7-azilithocholyl[2′-^{3}H(N)]taurine) using isolated hepatocytes and intact liver tissue. Photoaffinity labeling of isolated hepatocytes with [^{3}H]-7,7-ASLCT on the one hand and with [^{3}H]-7,7-ACT and [^{3}H]-7,7-ALCT on the other resulted in a different labeling pattern of cytosolic polypeptides, without a relevant incorporation of radioactivity into subunits of glutathione transferases. This suggests that glutathione transferases play no role in the transport of dianionic or of monoanionic bile salts. With [^{3}H]-7,7-ACT and [^{3}H]-7,7-ALCT a moderate incorporation of radioactivity was found in polypeptides with apparent M_rs of 33,000, 38,000, and 54,000, whereas with [^{3}H]-7,7-ASLCT, a polypeptide with an apparent M_r of 14,000, identified as H-FABP, was markedly and almost exclusively labeled. Photoaffinity labeling of specimens of intact liver tissue resulted in a labeling pattern of cytosolic polypeptides comparable to that obtained from photolabeled isolated hepatocytes. All results suggest that transcellular transport of dianionic sulfated as well as taurine-conjugated bile salts and of monoanionic taurine-conjugated bile salts follows different pathways. In intracellular transport of taurine-conjugated bile salts, several cytosolic polypeptides may have a function, whereas, in transport of taurine-conjugated 3α-sulfato bile salts, only H-FABP appears to be involved.—**Dietrich, A., W. Dieminger, K. Fuchte, G. H. Stoll, E. Schlitz, W. Gerok, and G. Kurz.** Functional significance of interaction of H-FABP with sulfated and nonsulfated taurine-conjugated bile salts in rat liver. *J. Lipid Res.* 1995. **36:** 1745–1755.

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Sulfated and taurine-conjugated bile salts are found in enterohepatic circulation mainly after application of the therapeutically administered bile salts chenodeoxycholate and ursodeoxycholate (1–3). The dianionic bile salts are taken up into hepatocytes by mediated transport, are not further metabolized intracellularly (4), and are reexcreted into bile. In sinusoidal uptake the dianionic sulfated and taurine-conjugated bile salt SLCT and the monoanionic taurine-conjugated bile salt cholyltaurine exhibit cross-inhibition suggesting that they share a common transport system (5), but their excretion into bile is mediated by different transport systems (6). In order to determine whether the dianionic and the monoanionic bile salts behave differently not only in canalicular excretion but also in intracellular transport, the photolabile derivative [^{3}H]-7,7-ASLCT was synthesized (4) and used in comparison with [^{3}H]-7,7-ACT and [^{3}H]-7,7-ALCT for the identification of binding polypeptides quite simply by photoaffinity labeling.

Bile salt binding polypeptides of cytosol have been detected so far only by studies using cytosolic fractions of liver and their physiological functions were inferred from studying their affinities to bile salts (7–14). However, studies with subfractions are of limited value and

Abbreviations: [^{3}H]-7,7-ACT, (7,7-azi-3α,12α-dihydroxy-5β-cholan-24-oyl)-2′-[2′-^{3}H(N)]aminoethanesulfonate; [^{3}H]-7,7-ALCT, 7,7-azilithocholyl[2′-^{3}H(N)]taurine or (7,7-azi-3α-hydroxy-5β-cholan-24-oyl)-2′-[2′-^{3}H(N)]aminoethanesulfonate; [^{3}H]-7,7-ASLCT, 7,7-azi-3α-sulfatolithocholyl[2′-^{3}H(N)]taurine or (7,7-azi-3α-sulfato-5β-cholan-24-oyl)-2′-[2′-^{3}H(N)]aminoethanesulfonate; [^{3}H]-11,11-azi-heptafluorostearate, 11,11-azi-2,2,3,3,18,18,18-heptafluoro[G-^{3}H]octadecanoate; H-FABP, hepatic fatty acid binding protein; heptafluorostearate, 2,2,3,3,18,18,18-heptafluorooctadecanoate; LSC, liquid scintillation counting; M_r, molecular weight; pI, isoelectric point; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SLCT, 3α-sulfatolithocholyltaurine or (3α-sulfato-5β-cholan-24-oyl)-2′-aminoethanesulfonate.

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do not allow us to draw conclusions about the physiological interactions in the unperturbated state. Because photoaffinity labeling experiments can be performed not only with cellular subfractions but also with isolated cells and intact tissue, this method is especially suitable for the detection of interactions in the unperturbated state (15). In the present study photoaffinity labeling of cytosol from liver, isolated hepatocytes, and intact liver tissue from rats has been used to provide evidence that dianionic and monoanionic bile salts follow intracellularly different pathways in the course of the hepatobiliary transport. The dianionic sulfated and taurine-conjugated bile salts interact intracellularly almost exclusively with H-FABP, so that this polypeptide is thought to have a function in the intracellular transport of sulfated and taurine-conjugated bile salts. Part of this work has been presented in preliminary form (5).

MATERIALS AND METHODS

Materials

Cholyltaurine was obtained from Sigma Chemie GmbH (Taufkirchen, Germany). [^{3}H]-7,7-ACT (750–1500 GBq/mmol), [^{3}H]-7,7-ALCT (750–1500 GBq/mmol), SLCT, [^{3}H]-7,7-ASLCT (750–1500 GBq/mmol), heptafluorostearate, and [^{3}H]-11,11-azi-heptafluorostearate (2,63 TBq/mmol) were synthesized as described (4, 16, 17). All other chemicals were of the highest quality available from commercial sources.

Animals

Male Wistar rats (Tierzuchtanstalt Jautz, Hannover, Germany) weighing 200–250 g were used. The animals had free access to 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.

Protein determination and detection of radioactivity

Protein concentration and radioactivity were determined as described (4). For detection of radioactivity by fluorographic analysis, hypersensitized films were used (18, 19).

Purification of glutathione transferases and H-FABP

All steps in the purification of the proteins were performed at 4°C unless otherwise stated.

Preparation of particle-free extract and separation of glutathione transferases and H-FABP. Livers were prepared from pentobarbital anesthetized rats (3 mg of sodium pentobarbital/100 g body weight, i.p.) after perfusion with physiological saline at 37°C. The minced livers (about 50 g) were homogenized in 150 mM sodium phosphate buffer, pH 7.4, (1.5 ml/g liver) by 10 strokes using a 60-ml glass homogenizer (Potter Elvehjem type tissue grinder, type S, Braun, Melsungen, Germany) with a Teflon pestle (clearance of 0.65 mm) turning at a speed of 1000 rev/min. The homogenate was centrifuged for 30 min at 23,000 *g* and the resulting supernatant was cleared by centrifugation for 60 min at 109,000 *g*. The clear supernatant (about 60 ml; 35–45 mg protein/ml) was aspirated carefully avoiding contamination with the lipid layer and subsequently applied to a column of Sephadex G-75 SF (5 × 100 cm) equilibrated with 30 mM Tris/HCl buffer, pH 9.0, containing 100 mM NaCl and 1 mM EDTA. Fractions of 5 ml were collected at a flow rate of 2 ml/cm^2 per h.

Isolation of glutathione transferase 1–1 (Y_aY_a) and 1–2 (Y_aY_c) (20). Fractions containing enriched glutathione transferases (M_r of subunit 1 (Y_a): 26,000; M_r of subunit 2 (Y_c): 28,000), as revealed by SDS-PAGE, were combined and the glutathione transferases 1–1 and 1–2 were isolated from this protein solution principally following the procedures described (21–23).

Isolation of H-FABP. Fractions containing enriched H-FABP (M_r: 14,000), as revealed by SDS-PAGE, were combined and the resulting protein solution was concentrated to a final volume of about 20 ml (80–100 mg protein) by ultrafiltration using a YM 5 membrane (Amicon GmbH, Witten, Germany). The protein solution was equilibrated against 10 mM sodium phosphate buffer, pH 6.8, by chromatography on a column of Sephadex G-25 M (2.5 × 50 cm) and subsequently applied to a hydroxyapatite column (3 × 20 cm) preequilibrated with the same buffer. The column was washed with 300 ml of 10 mM sodium phosphate buffer, pH 6.8. After removing contaminating protein with 800 ml of 10 mM sodium phosphate buffer, pH 6.8, containing 500 mM NaCl, H-FABP was eluted with 800 ml of 10 mM sodium phosphate buffer, pH 6.8, containing 1 M NaCl. Fractions of 10 ml were collected at a flow rate of 60 ml/h. Fractions revealing no impurities by SDS-PAGE were combined, equilibrated with 30 mM Tris/HCl buffer, pH 9.0, and concentrated to a final volume of about 5 ml by ultrafiltration using a YM 5 membrane.

The purified protein (about 25 mg) was stored in 30 mM Tris/HCl buffer, pH 9.0, containing 10% glycerol at −80°C.

Preparation of liver snips

Rat livers were perfused in situ at 37°C with a standard medium consisting of 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, which was saturated with carbogen (95% O_2/5% CO_2) and adjusted to pH 7.4. The pressure during perfusion was maintained at 12 ± 0.5 cm water pressure. After 10

min of perfusion, liver snips with 1 to 2 mm in cross section and 2 to 4 mm in height were excised by a pair of scissors used for iridectomy (No. H-4265, Heiss, Augeninstrumente, Tuttlingen, Germany) while maintaining the perfusion. In order to get representative probes of the whole liver, snips were excised from the edge and from the center of each lobe. Immediately after excision the liver snips were kept in standard medium at 37°C under an atmosphere of carbogen with gentle shaking.

Isolation of hepatocytes

Isolation and characterization of hepatocytes were performed exactly as described (24).

Isolation of cytosol

Cytosol from isolated hepatocytes was obtained by freezing and thawing of hepatocytes three times using liquid nitrogen or by intracytoplasmic cavitation of nitrogen gas (25, 26) with a gas pressure of 4.5 MPa at 4°C using a pressure homogenizer (Kontes Europe, Carnforth, England). The homogenate was centrifuged for 45 min at 175,000 *g* and the resulting supernatant was used for further experiments.

Cytosol from liver snips was obtained by homogenization of 10–30 liver snips in 1–3 ml of the appropriate buffer by 14 strokes of a 15-ml glass homogenizer (Dounce type tissue grinder, type S, Braun, Melsungen, Germany) with a tight-fitting pestle. The resulting homogenate was centrifuged for 30 min at 200,000 *g*. The clear supernatant was aspirated carefully, avoiding contamination with the lipid layer, and used for further experiments.

Photoaffinity labeling

Photoaffinity labeling of isolated hepatocytes, performed in order to identify bile salt binding polypeptides, was carried out in 1 ml of a hepatocyte suspension (about $1–2 \times 10^6$ cells/ml, 2–4 mg protein/ml) containing 1–10 μM of the 7,7-azi derivative of the appropriate bile salt or 0.1–1 μM of the [^{3}H]-11,11-azi-heptafluorostearate at 30°C as described (27). Photoaffinity labeling of liver snips was carried out principally as described (15) after incubation of 10–30 liver snips with 4 μM of [^{3}H]-7,7-ASLCT in 1 ml of standard medium for 30 min in the dark at 37°C under an atmosphere of carbogen with gentle shaking. Neither the hepatocyte incubation solution nor the liver-snip solution contained albumin or other proteins. Irradiation was performed at 30°C for 15 min. The incubation medium was removed and the liver snips were washed two times with 0.5 ml of standard medium. The liver snips were freed from impaired cells by incubation with 2 ml of standard medium containing 150 μg/ml trypsin and 100 μg/ml DNAse for 5 min at 37°C. Enzymatic digestion was stopped by removal of the incubation medium and subsequent treatment with 3 ml of standard medium containing 300 μg/ml trypsin inhibitor. After 3 min incubation at 37°C, the liver snips were washed two times with 0.5 ml of 1 mM $NaHCO_3$, pH 7.4, each.

Polyacrylamide gel electrophoresis and isoelectric focusing

Discontinuous SDS-PAGE using vertical slab gels (200 × 180 × 2.8 mm) was performed as described (28). Isoelectric focusing was carried out on Ampholine gel PAGplates pH 3.5–9.5 (Pharmacia LKB, Freiburg, Germany). pI values were determined by the use of pI marker proteins (Broad calibration kit, Pharmacia LKB, Freiburg, Germany) and controlled by cutting out zones of gel, eluting the latter with 10 mM KCl and measuring the pH values.

Production and purification of antibodies

Antibodies were raised in chinchilla bastard rabbits by application of 4 mg of purified protein mixed with 0.4 ml $Al(OH)_3$ suspension and 0.4 ml complete Freund's adjuvant. After 1 month the procedure was repeated using incomplete Freund's adjuvant. The IgG fraction of the antiserum was prepared by ammonium-sulfate fractionation.

Immunoprecipitation

Immunoprecipitation was performed as described previously (29). However, protein probes were solubilized with 0.5% Triton X-100 and antigen–antibody incubation was carried out with a Triton X-100 concentration diluted to 0.1%.

RESULTS AND DISCUSSION

Identification of bile salt-binding polypeptides in the soluble fraction of rat liver

Bile salt binding by cytosolic proteins is assumed to be involved in intracellular translocation of bile salts on their path from sinusoidal to canalicular membrane of the hepatocyte (30). The proteins considered as having an intracellular function in bile salt transport comprise the glutathione transferases (31–34), bile salt binding polypeptides with apparent M_rs about 33,000 (8), identified as 3α-hydroxysteroid dehydrogenases (35), and a polypeptide with an apparent M_r of 14,000, probably H-FABP (11).

Hepatobiliarily transported bile salts differ in their structure and function. Whereas the monoanionic glycine- or taurine-conjugated bile salts exert physiological functions in lipid digestion and resorption, the dian-

ionic sulfated and glycine- or taurine-conjugated bile salts are detoxication products. Consequently, biliary secretion of monoanionic and excretion of dianionic bile salts is mediated by different canalicular transport systems (6, 36). Thus, the idea that monoanionic and dianionic bile salts use intracellularly different pathways was worth examining. In order to identify the soluble polypeptides that interact with SLCT, the soluble fraction obtained from rat liver was submitted to photoaffinity labeling, using [^{3}H]-7,7-ASLCT as photolabile derivative. Subsequent analysis by SDS-PAGE revealed a clear incorporation of radioactivity in polypeptides with the apparent M_rs of 26,000, 28,000, 54,000, and 67,000 (**Fig. 1A**). The polypeptide with the apparent M_r of 26,000, by far the predominant labeled one, was identified by immunoprecipitation with polyclonal antibodies against glutathione transferase 1–1 (also designated as Y_aY_a) as well as against glutathione transferase 1–2 (also designated as Y_aY_c) as subunit 1 of glutathione transferase (Fig. 1B). The coprecipitation of the slightly labeled polypeptide with the apparent M_r of 28,000 by both antibodies demonstrates that subunit 2 of glutathione transferases also has the capability to interact with [^{3}H]-7,7-ASLCT. The identification of glutathione transferases as SLCT-binding proteins by photoaffinity labeling is in accordance with the results obtained by binding studies with purified glutathione transferases (34).

In order to compare the SLCT-binding polypeptides of liver cytosol with those interacting with monoanionic bile salts, photoaffinity labeling of the soluble fraction obtained from rat liver was performed using [^{3}H]-7,7-ACT as a photolabile derivative of cholyltaurine. The pattern of labeled polypeptides obtained after SDS-PAGE was dependent on the method of liver disintegration and differs from that resulting from photoaffinity labeling experiments with [^{3}H]-7,7-ASLCT (Fig. 1C). Incorporation of radioactivity occurred in polypeptides with the apparent M_rs of 14,000, 26,000, 28,000, 33,000, 38,000, 43,000, 54,000, and 67,000. The polypeptides with the apparent M_rs of 26,000 and 28,000, identified by immunoprecipitation as subunits 1 and 2 of glutathione transferases, exhibited the strongest labeling together with the polypeptide with the apparent M_r of 38,000. The labeled polypeptide with the M_r of 43,000 is the subunit of hydroxycholanoyltransferase (H. Abberger and G. Kurz, unpublished results) and that with the M_r of 67,000 is albumin. The labeling of a polypeptide in the M_r range about 33,000 may be caused by proteins of the family of bile acid binders (8, 30, 35) and that of the smallest polypeptide with the apparent M_r of 14,000 may be due to a bile salt-binding protein of the Z-fraction (11, 30). The appearance of the labeled polypeptide with the apparent M_r of 54,000 is dependent on the effectivity of disintegration of liver, fitting with its localization as a peripheral membrane protein of endoplasmic reticulum (A. Stolz and G. Kurz, unpublished results).

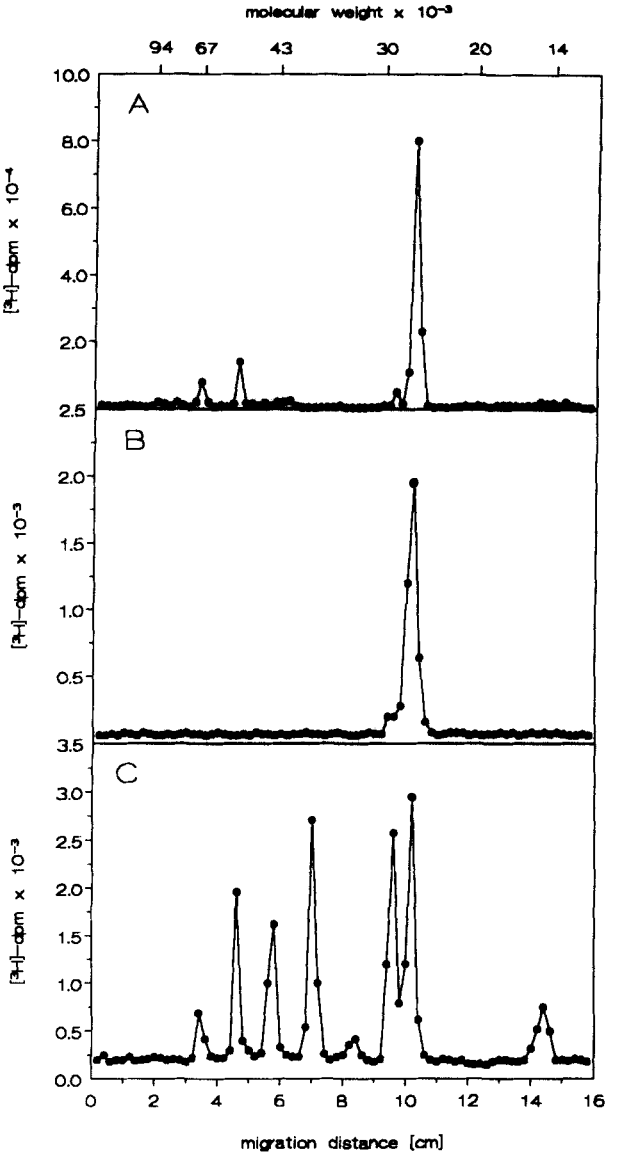

Fig. 1. Identification of bile salt-binding polypeptides of cytosol from rat liver. Distribution of radioactivity after SDS-PAGE of cytosol. A solution of 400 μg of cytosol in standard medium was incubated with 1 μM [^{3}H]-7,7-ASLCT (370 kBq) or 1 μM [^{3}H]-7,7-ACT (370 kBq) at 30°C for 5 min and irradiated for 10 min with light having a maximum at 350 nm. Total acrylamide concentration of the gel was 12% at a ratio of acrylamide–bisacrylamide of 97.2:2.8. A: Distribution of radioactivity after photoaffinity labeling of cytosol with [^{3}H]-7,7-ASLCT; B: distribution of radioactivity in the immunoprecipitate obtained subsequent to photoaffinity labeling of cytosol with [^{3}H]-7,7-ASLCT by precipitation with antibodies against glutathione transferase 1–2 (29). C: Distribution of radioactivity after photoaffinity labeling of cytosol with [^{3}H]-7,7-ACT.

Identification of cytosolic SLCT-binding polypeptides in isolated hepatocytes

Photoaffinity labeling of the cytosolic fraction of liver resulted in the identification of bile salt-binding polypeptides and probably the same polypeptides that were identified by binding studies using hepatic cytosol or purified proteins were found (30). However, to draw conclusions from these experiments about the involvement of the identified polypeptides in intracellular transport is not admissible. Because the biological significance of investigations requiring the disruption of the cellular organization is restricted with regard to the native state, intracellular transport processes should be studied in systems where the morphological and functional integrity is preserved (15). Based on this fundamental condition, only particular methods are suitable for the study of intracellular transport processes. Photoaffinity labeling of intact cells and tissues is such a suitable chemical method because the labeling reactions precede the disintegration of the intact biological systems. Thus, in order to obtain clear information about the interactions occurring in vivo, photoaffinity labeling studies with both isolated hepatocytes and tiny snips of intact liver were performed.

Photoaffinity labeling of freshly prepared isolated hepatocytes using [^{3}H]-7,7-ASLCT in the concentration range of 1 to 10 μM and subsequent analysis of total cell protein by SDS-PAGE revealed a labeling pattern (**Fig. 2A**) completely different from that obtained by labeling of isolated cytosol (Fig. 1A). Radioactivity was found predominantly incorporated into a polypeptide with the apparent M_r of 14,000 and compared with the labeling of this polypeptide that of other polypeptides was of minor importance. Fifty-two percent of total incorporated radioactivity was bound to this polypeptide. In order to determine whether the labeled polypeptide was a cytosolic one or was associated with membranous cell organelles, gentle disintegration of labeled hepatocytes was performed by pressure homogenization (25, 26) or by freezing and thawing, taking care to cause least damage to the cellular organelles. Subsequent analysis of the cytosolic fraction demonstrated that the labeled polypeptide with the apparent M_r of 14,000 was a cytosolic one (Fig. 2B). In addition to this polypeptide in cytosol of photolabeled hepatocytes, only polypeptides with the apparent M_rs of 26,000 and 67,000 were found labeled to a negligible extent. The labeling of these polypeptides may be due to the small amount of nonviable hepatocytes not avoidable in all cell preparations.

Identification of cytosolic SLCT-binding polypeptides in intact liver tissue

Because isolated hepatocytes have lost their structural polarity, at least partially, and their capability for canalicular bile salt secretion (15, 24), results obtained with isolated hepatocytes may not be valid for intact liver. In order to identify the intracellular SLCT-binding polypeptides in hepatocytes having an intact hepatobiliary transport, tiny liver snips were submitted to photoaffinity labeling. The hepatocytes of the outer cell layers of these liver snips exhibit an intact hepatobiliary transport of bile salts qualitatively and are therefore suitable for the identification of transport processes requiring the polarity of the cells. In order to analyze only the results obtained with intact hepatocytes, the impaired cells on the surface of the snips were removed to a wide extent by enzymatic digestion (15). The pattern of labeled polypeptides obtained from total cell protein after photoaffinity labeling of snips with 1–10 μM [^{3}H]-7,7-ASLCT showed the highest incorporation of radioactivity into a polypeptide with the apparent M_r of 14,000 (**Fig. 3A**). The labeling pattern of the cytosolic fraction (Fig. 3B) obtained from liver snips subsequent to photoaffinity labeling was similar to that obtained

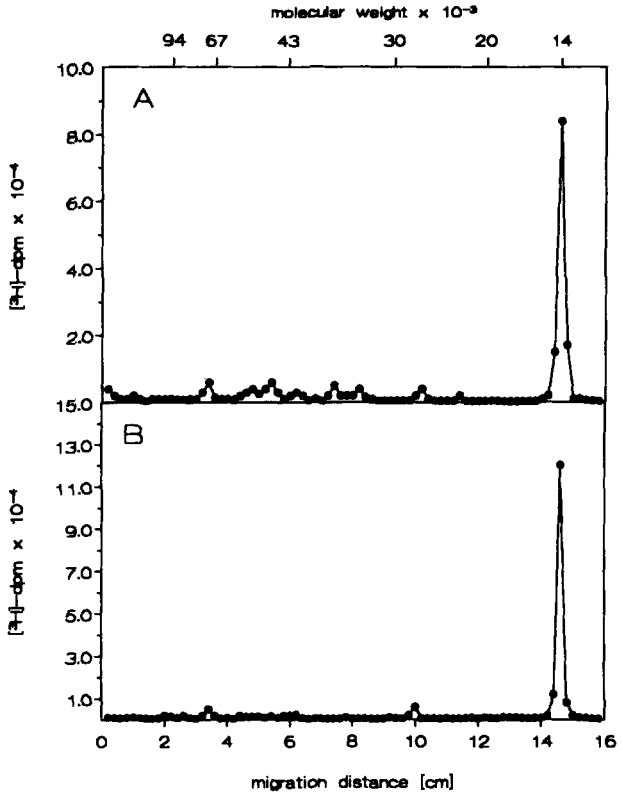

Fig. 2. Identification of SLCT-binding polypeptides in isolated hepatocytes. Distribution of radioactivity after SDS-PAGE of freshly isolated hepatocytes subsequent to photoaffinity labeling. A cell suspension of 1 ml containing about 1 × 10^6 (2 mg protein) hepatocytes in standard medium was incubated with 1 μM [^{3}H]-7,7-ASLCT (370 kBq) at 37°C for 10 min and subsequently submitted to photoaffinity labeling. All other conditions were as described in the legend to Fig. 1. A: Total cell protein (500 μg); B: cytosolic protein (500 μg).

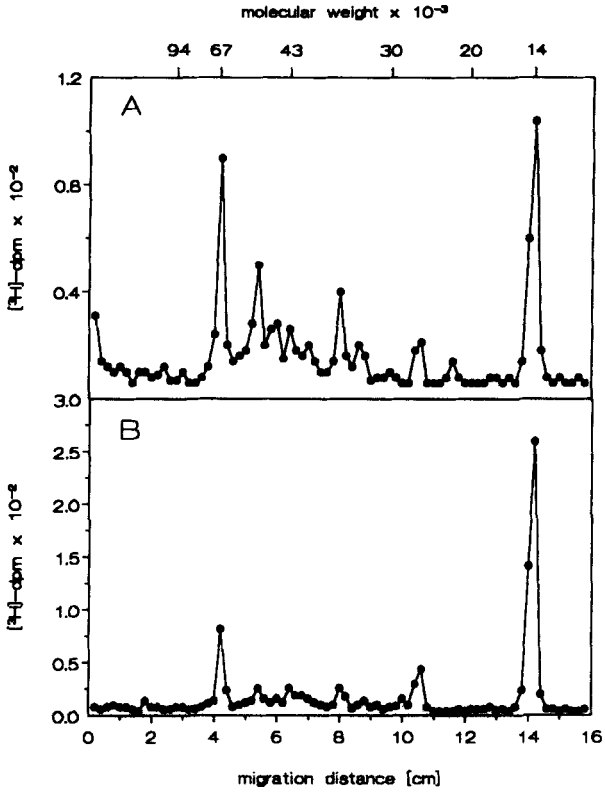

Fig. 3. Identification of SLCT-binding polypeptides in intact liver tissue. Distribution of radioactivity after SDS-PAGE. Ten liver snips were preincubated with 4 μM [^{3}H]-7,7-ASLCT (1.5 MBq) in standard medium at 37°C for 30 min and irradiated at 30°C for 15 min. SDS-PAGE was performed using an acrylamide gel gradient from 10–15%. A: Total protein of liver snips (450 μg) after removal of impaired cells by enzymatic digestion; B: cytosolic protein from liver snips (450 μg).

with cytosol from labeled isolated hepatocytes (Fig. 2B). Only the same polypeptides were found labeled and it is obvious that again the polypeptide with the apparent M_r of 14,000 is the predominantly labeled one. The relatively low labeling of a polypeptide with the apparent M_r of 26,000, resulting once more from the subunit of a glutathione transferase, was probably due to the fact that removal of impaired cells was practically always incomplete. The labeling of a polypeptide with the M_r of 67,000 came from rat serum albumin, as demonstrated by immunoprecipitation. Albumin was found labeled to varying extents and originated probably from traces of serum within the snips, not completely removed by perfusion of liver rather than from intracellular sources.

The photoaffinity labeling studies with [^{3}H]-7,7-ASLCT using isolated hepatocytes and intact liver tissue were consistent with the conclusion that a cytosolic polypeptide with the M_r of 14,000 was involved in intracellular transport of sulfated and taurine conjugated bile salts. These data suggest that intracellular binding of dianionic bile salts by other polypeptides or proteins, especially by glutathione transferases, is of little or no biological significance.

Identity of the SLCT-binding polypeptide with H-FABP

In order to characterize the SLCT-binding polypeptide, its M_r under nondenaturating conditions was first estimated by gel permeation chromatography on a Sephadex G-75 column to be about 14,000 (not shown). This proves that the polypeptide is not a component of a composed protein but exists in vivo as a monomer and suggests it is a binding protein of the Z-fraction, probably H-FABP. Therefore, comparative studies were performed using the photolabile derivative of a metabolically stable long-chain fatty acid salt, the suitability of which had been demonstrated by photoaffinity labeling of isolated H-FABP (17). Photoaffinity labeling of isolated hepatocytes with 0.1–1 μM of [^{3}H]-11,11-azi-heptafluorostearate and subsequent analysis of the cytosolic fraction by SDS-PAGE showed that under the conditions used, incorporation of radioactivity occurred predominantly in a polypeptide with the M_r of 14,000 (**Fig. 4**). Only two other polypeptides with apparent M_rs of about 30,000 and 67,000 were slightly labeled.

As a further criterion for the identity of the polypeptide photoaffinity labeled either by [^{3}H]-7,7-ASLCT or by [^{3}H]-11,11-azi-heptafluorostearate, the pI values were

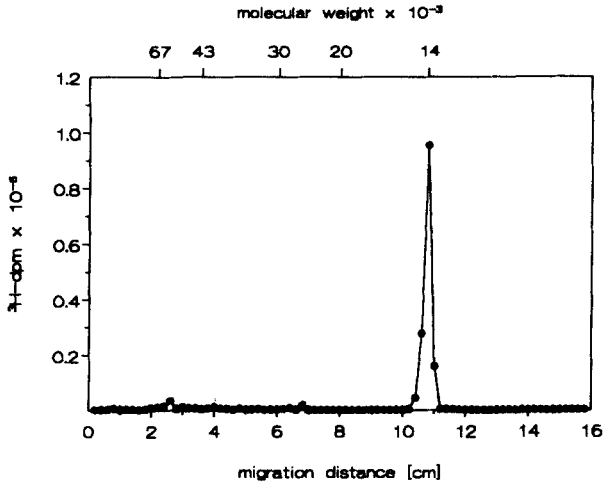

Fig. 4. Identification of H-FABP in cytosol of isolated hepatocytes. Distribution of radioactivity after SDS-PAGE of cytosol obtained from freshly isolated hepatocytes subsequent to photoaffinity labeling with [^{3}H]-11,11-azi-heptafluorostearate. A cell suspension of 0.5 ml containing about 1×10^6 (2 mg protein) hepatocytes in standard medium was incubated with 0.2 μM [^{3}H]-11,11-azi-heptafluorostearate (280 kBq) at 37°C for 10 min and subsequently irradiated. Total acrylamide concentration of the gel was 15% at a ratio of acrylamide–bisacrylamide of 97.2:2.8; 400 μg of cytosolic protein was applied to electrophoresis. All other conditions were as described in the legend to Fig. 1.

used as a comparison. The separation of total cytosolic proteins obtained from isolated hepatocytes, subsequent to photoaffinity labeling by isoelectric focusing in polyacrylamide gels using a pH gradient from 3.5 to 9.5, revealed that the cytosolic polypeptides labeled with either [^{3}H]-7,7-ASLCT or [^{3}H]-11,11-azi-heptafluorostearate appear at the same pI of 5.4 (**Fig. 5**). The identification of both labeled polypeptides with regard not only to their M_r but also to their pI makes it highly probable that the SLCT-binding polypeptide is identical to H-FABP. In order to prove this, isolated H-FABP (**Fig. 6A**) was submitted to differential photoaffinity labeling. The extent of photoaffinity labeling of H-FABP using [^{3}H]-7,7-ASLCT was clearly decreased in the presence of stearate as competing ligand (Fig. 6B) and, vice versa, incorporation of radioactivity by photoaffinity labeling using [^{3}H]-11,11-azi-heptafluorostearate was diminished in the presence of SLCT (Fig. 6C). These results demonstrate unequivocally that sulfated and taurine-conjugated bile salts and long-chain fatty acid salts compete

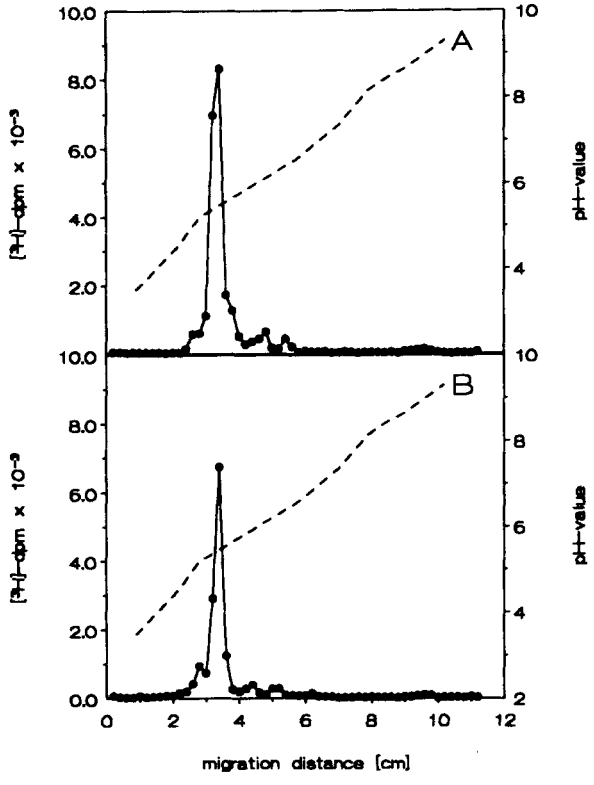

Fig. 5. Distribution of radioactivity after isoelectric focusing of cytosol from freshly isolated hepatocytes subsequent to photoaffinity labeling. A cell suspension of 0.5 ml containing about 1×10^6 (2 mg protein) hepatocytes in standard medium was incubated with 1 μM [^{3}H]-7,7-ASLCT (370 kBq) or with 0.2 μM [^{3}H]-11,11-azi-heptafluorostearate (280 kBq) at 37°C for 10 min and subsequently irradiated. Forty μg of cytosolic protein was applied to isoelectric focusing. A: Photoaffinity labeling with [^{3}H]-7,7-ASLCT; B: photoaffinity labeling with [^{3}H]-11,11-azi-heptafluorostearate.

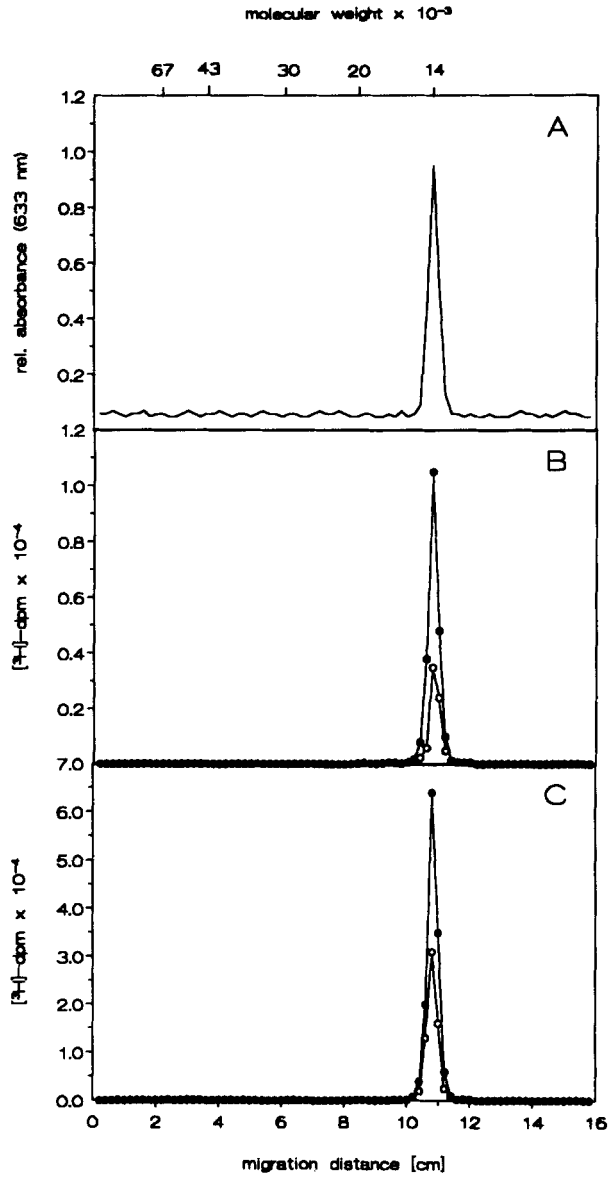

Fig. 6. Differential photoaffinity labeling of isolated H-FABP. Photoaffinity labeling of 30 μg of the purified protein was performed either with 1 μM [^{3}H]-7,7-ASLCT (370 kBq) in the absence and in the presence of 50 μM stearate or with 0.4 μM [^{3}H]-11,11-azi-heptafluorostearate (110 kBq) in the absence and in the presence of 100 μM SCLT. Total acrylamide concentration of the gel was 15%. All other conditions were as described in the legend to Fig. 1. A: Relative absorbance at 633 nm after staining with Coomassie Brilliat Blue R; B: distribution of radioactivity after photoaffinity labeling with [^{3}H]-7,7-ASLCT in the absence (●) and in the presence (○) of stearate; C: distribution of radioactivity after photoaffinity labeling with [^{3}H]-11,11-azi-heptafluorostearate in the absence (●) and in the presence (○) of SLCT.

for the same binding sites of the very same polypeptide H-FABP.

The binding of sulfated and taurine-conjugated bile salts on the one hand and of long-chain fatty acid salts on the other hand induces the same shift in the pI of the

polypeptide (**Fig. 7**). Isolated H-FABP, migrating as a single component in polyacrylamide gel electrophoresis in the absence and in the presence of SDS, did not show any sign of microheterogeneity and exhibited the pI of 7.35 (Fig. 7A). After photoaffinity labeling of this polypeptide with either [^{3}H]-7,7-ASLCT or [^{3}H]-11,11-azi-heptafluorostearate, the labeled polypeptide appeared exclusively at the pI of 5.4 (Figs. 7B and 7C). The shift of pI obtained upon photoaffinity labeling of H-FABP corresponds to the shift observed by nonconvalent binding of salts of long-chain fatty acids to this polypeptide (37, 38). To a lesser extent, such a shift of pI is also found upon binding of salts of long-chain fatty acids to serum albumin (39, 40). The same marked shift of the pI of the polypeptide after binding the ligands is an indication that interaction with both sulfated and taurine-conjugated bile salts and long-chain fatty acid salts induces a similar conformational change.

The identity of the polypeptide labeled by [^{3}H]-7,7-ASLCT with H-FABP was further demonstrated by immunoprecipitation with monospecific antibodies against the purified H-FABP (**Fig. 8**) and partial amino acid sequence analysis. Amino acid sequence determination of four peptides isolated after tryptic digestion of the isolated polypeptide allowed alignment of 73 of the 127 amino acids to the known sequence (41, 42). The entirety of results proves that the cytosolic peptide that

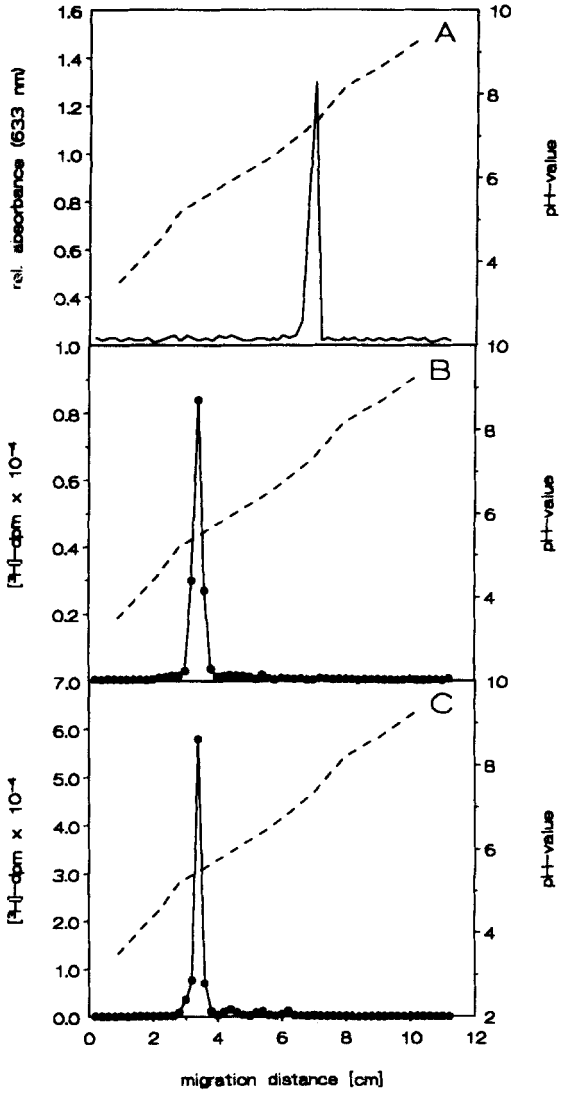

Fig. 7. Isoelectric focusing of purified H-FABP after photoaffinity labeling. Photoaffinity labeling of 30 μg of isolated H-FABP was performed either with 1 μM [^{3}H]-7,7-ASLCT (370 kBq) or with 0.4 μM [^{3}H]-11,11-azi-heptafluorostearate (110 kBq). All other conditions were as described in the legend to Fig. 1. For isoelectric focusing 15 μg of the labeled protein was applied to the gel plates. A: Relative absorbance at 633 nm after staining with Coomassie Brilliant Blue R; B: distribution of radioactivity after photoaffinity labeling with [^{3}H]-7,7-ASLCT; C: distribution of radioactivity after photoaffinity labeling with [^{3}H]-11,11-azi-heptafluorostearate.

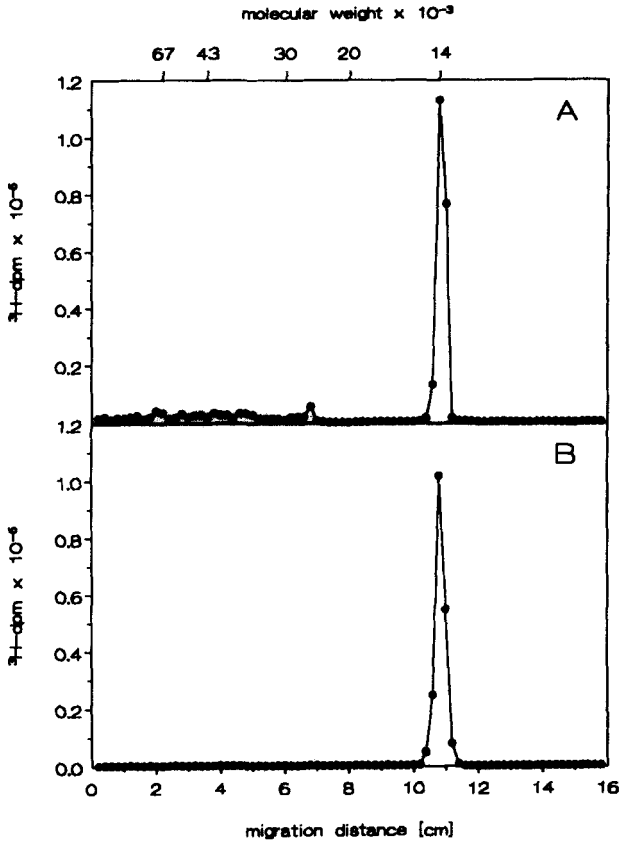

Fig. 8. Immunoprecipitation of H-FABP. Cytosol of freshly isolated hepatocytes subsequent to photoaffinity labeling with 1 μM [^{3}H]-7,7-ASLCT (370 kBq) was used for immunoprecipitation of H-FABP. All conditions were as described in the legend to Fig. 2. Immunoprecipitation was performed as described previously (29). Total acrylamide concentration of the gel was 15%. A: Distribution of radioactivity after SDS-PAGE of cytosolic proteins; B: distribution of radioactivity after SDS-PAGE of the immunoprecipitate obtained with antibodies against H-FABP.

binds sulfated and taurine-conjugated bile salts corresponds to H-FABP.

Function of H-FABP in intracellular bile salt transport

The labeling patterns of polypeptides obtained by photoaffinity labeling of isolated hepatocytes and liver snips strongly indicate that sulfated and taurine-conjugated bile salts interact in the course of their transport from sinusoidal to canalicular membrane practically only with H-FABP, which therefore must have a function in storage and/or transport. H-FABP also has the ability to bind monoanionic taurine-conjugated bile salts, as demonstrated by photoaffinity labeling of the soluble fraction obtained from liver using [^{3}H]-7,7-ACT (Fig. 1C). The simplest assumption that H-FABP is the main intracellular binding protein not only for the dianionic but also for the monoanionic bile salts, was examined by comparative photoaffinity labeling of isolated hepatocytes. [^{3}H]-7,7-ASLCT was used as a photolabile derivative of dianionic bile salts and [^{3}H]-7,7-ACT as well as [^{3}H]-7,7-ALCT were used as derivatives of monoanionic bile salts. Photoaffinity labeling of isolated hepatocytes with these three photolabile derivatives and subsequent analysis of the cytosolic proteins by SDS-PAGE revealed clear qualitative and, even more eye catching, quantitative differences between the labeling patterns obtained with [^{3}H]-7,7-ASLCT on the one hand and those with [^{3}H]-7,7-ACT and [^{3}H]-7,7-ALCT on the other hand (**Fig. 9**). Photoaffinity labeling using [^{3}H]-7,7-ASLCT caused the predominant labeling of H-FABP (Fig. 9, lane B), a slight labeling of a polypeptide with M_r of about 26,000, and a still slighter labeling of one with the M_r of 67,000. The slight labeling of the polypeptide with the M_r about 26,000 occurred to varying extents and seems to parallel the decrease of viability of the hepatocyte populations. Whereas with hepatocyte preparations having a viability of > 92% labeling of this polypeptide is about 5% as compared to that of H-FABP, its labeling increases with decreasing cell viability. Thus, incorporation of radioactivity into the polypeptide with the M_r about 26,000 was considered to be insignificant and its participation in transport of dianionic bile salts seemed to be unlikely.

Photoaffinity labeling of isolated hepatocytes using [^{3}H]-7,7-ACT (Fig. 9, lane A) and [^{3}H]-7,7-ALCT (Fig. 9, lane C) resulted in different labeling patterns and it was evident that total incorporation of radioactivity into polypeptides of the cytosolic fraction of hepatocytes was considerably lower than that obtained with 7,7-ASLCT. This shows that monoanionic taurine-conjugated bile salts and dianionic sulfated and taurine-conjugated bile salts behave differently in intracellular hepatic transport. The different labeling patterns and intensities

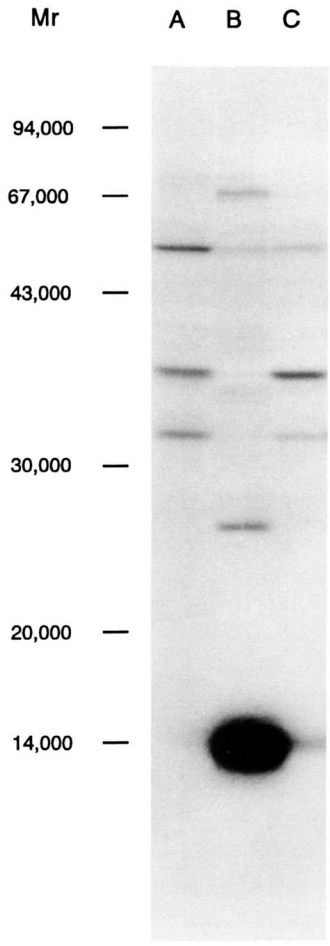

Fig. 9. Fluorographic identification of bile salt-binding polypeptides in the cytosol after photoaffinity labeling of freshly isolated hepatocytes. A cell suspension of 1 ml containing about 2×10^6 (4 mg protein) hepatocytes in standard medium was incubated with 1 μM (370 kBq) of the respective photolabile derivative. Subsequent to the photoaffinity labeling procedure the cells were disintegrated by freezing and thawing three times. The homogenates were freed from particulate material by high speed centrifugation and 300 μg of cytosolic proteins from the supernatants was applied to each lane of the gel. Total acrylamide concentration was 12%. All other conditions were as described in the legend to Fig. 2. Lane A: Photoaffinity labeling with [^{3}H]-7,7-ACT; lane B: photoaffinity labeling with [^{3}H]-7,7-ASLCT; lane C: photoaffinity labeling with [^{3}H]-7,7-ALCT.

obtained with photolabile derivatives of monoanionic and dianionic bile salts (Fig. 9), respectively, may be easily explained with the assumptions that a vesicular component may play a distinct role in transcellular traffic of monoanionic bile salts (43–47) and that dianionic bile salts pass through hepatocytes interacting with H-FABP.

Labeling of H-FABP with the photolabile derivatives of both monoanionic bile salts occurred to a very slight extent; only the polypeptides with the apparent M_rs of 33,000, 38,000, and 54,000 were found labeled to a

higher extent (Fig. 9, lane A and C). According to this, a function of these polypeptides in intracellular transport of monoanionic bile salts may be taken into consideration (9, 30). Photoaffinity labeling of isolated hepatocytes with the 7,7-azi derivatives of both monoanionic taurine-conjugated bile salts resulted in practically no labeling of polypeptides with the M_r about 26,000, ruling out the involvement of glutathione transferases in intracellular transport of monoanionic bile salts. Thus, with respect to the role of cytoplasmic proteins in hepatic transport of both dianionic and monoanionic bile salts, an involvement of the glutathione transferases must be virtually excluded. Although the glutathione transferases have the ability to bind bile salts, the morphological and functional organization of the intact hepatocyte in liver guarantees that under physiological conditions bile salts do not interact with the glutathione transferases. Even the inhibition of their enzymatic activity by bile salts (48–50) should be of no relevance under physiological conditions.

Because functioning intracellular transport is dependent on the integrity of the morphological organization of hepatocytes, it is to be expected that examination methods starting with biological material of different levels of complexity end up with differing results. This is convincingly demonstrated in the identification of different bile salt-binding polypeptides by photoaffinity labeling of cytosol from rat liver (Fig. 1) and of isolated hepatocytes (Fig. 2) or intact liver tissue (Fig. 3) using the same photolabile derivatives. Photoaffinity labeling can be performed with different levels of biological organization, with subcellular fractions, with isolated cells, and intact liver tissue. It is a method that fixes the transient interactions occurring during intracellular or transcellular transport by covalent bond formation. This fixation of interactions in intact biological material precedes the disruption of the cells necessary for further analysis. Thus, despite the necessity to destroy the cellular structure, clear information about interactions occurring in vivo may be obtained.

However, conclusions drawn from photoaffinity labeling studies with intact cells or tissue depend on the analogy of a photolabile derivative and its biological original compound and, in the case of hepatobiliary bile salt transport, results could be misleading if the 7,7-azi analogues do not faithfully trace the corresponding original bile salt. A comparison of the behavior of 7,7-ASLCT and SLCT in the course of their hepatobiliary transport indicated that both dianionic bile salts are true competing substrates (4). On this basis it may be concluded that dianionic and monoanionic bile salts use different paths within the hepatocyte during their hepatobiliary transport, and that for sulfated and taurine-conjugated bile salts H-FABP is the essential intracellular binding protein.

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