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Identification and Comparison of Bile Acid-Binding Polypeptides in Ileal Basolateral Membrane

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Summary. Bile acid-binding polypeptides were examined using basolateral membrane vesicles and enterocytes isolated from rat ileum. The uptake of a photolabile taurocholate derivative, (7,7,-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonate,7,7-azo-TC, in ileal vesicles preloaded with paraaminohippurate (PAH) was stimulated with respect to uptake in unpreloaded vesicles. The PAH-*trans*stimulated uptake of 7,7-azo-TC was inhibited by taurocholate and vice versa. Irradiation of membrane vesicles in the presence of 7,7-azo-TC irreversibly inhibited PAH-*trans*stimulated taurocholate uptake. Photoaffinity labeling of basolateral membrane vesicles directly with [^{3}H] 7,7-azo-TC and separation of proteins by SDS-PAGE revealed incorporation of radioactivity into several polypeptides. Photoaffinity labeling of vesicles in the presence of taurocholate inhibited the labeling of 54,000 and 59,000 mol. wt. polypeptides. The efflux of taurocholate from ileal enterocytes was *cis*-inhibited by 7,7-azo-TC and *trans*stimulated by PAH. Irradiation of enterocytes in the presence of 7,7-azo-TC inhibited taurocholate efflux greater than the presence of 7,7-azo-TC in the dark. When enterocytes that were irradiated in the presence of [^{3}H] 7,7-azo-TC were fractionated and the resultant basolateral membrane fraction was subjected to SDS-PAGE, incorporation of radioactivity into the 54,000 and 59,000 mol. wt. polypeptides was seen. In contrast, when the brush-border membrane fraction was subjected to SDS-PAGE, greatest incorporation of radioactivity was seen in the previously described 99,000 mol. wt. polypeptide. These studies suggest that 7,7-azo-TC shared transporters with natural bile acid and identified polypeptides that may be involved in bile acid transport across the basolateral membrane and differ from that seen in the brush-border membrane of the ileal epithelial cell.

Key Words bile acid-binding polypeptides · basolateral membrane · photoaffinity labeling

Introduction

Bile acids are sterol molecules that have the property to form micelles, which accounts for their solubilization of lipolytic products of digestion in the intestinal lumen and of lipid products secreted by the liver into bile. To maintain adequate concentrations for micellarization, bile acids undergo an enterohepatic circulation; *i.e.*, they are synthesized in the liver, secreted into bile, emptied into the small intestine where they are absorbed, and gain entry to the liver via the portal circulation [21]. Previous in vivo studies in man [9] and laboratory animals [26] and in vitro studies with intestinal segments [5, 7, 16, 26, 34], isolated epithelial cells [35, 37] and brush-border membrane vesicles [2, 15, 19, 36] indicated that bile acids are transported across the small intestinal mucosal membrane either by ionic or nonionic passive diffusion and, in addition, across the ileal brush-border membrane by an active transport process. The energy for active bile acid absorption is derived from the Na^+ electrochemical gradient across the brush-border membrane. The Na^+ gradient arises from the extrusion of Na^+ in exchange for K^+ by Na^+-K^+-ATPase located on the basolateral membrane. A putative transport protein involved in coupled Na^+-bile acid transport across the ileal brush-border membrane has been identified as a 99,000 mol. wt. polypeptide using photoaffinity labeling techniques [3, 12].

In contrast to our knowledge of events involved in the uptake of bile acids by the brush-border membrane, recent studies suggest that a mechanism for the exit of bile acids from the intestinal epithelial cell across the basolateral membrane is anion exchange [30]. The purpose of the present studies is to identify and compare a binding polypeptide(s) that may be involved in the basolateral membrane transport of bile acid. Using basolateral membrane vesicles and enterocytes isolated from the rat ileum, affinity labeling studies were performed with a pho-

tolabile derivative of taurocholate, 7-7-azo-TC[1]. These studies suggest that 54,000 and 59,000 mol. wt. polypeptides may be involved in the exit of bile acids from the ileal epithelial cell.

Materials and Methods

Basolateral Membrane Preparation

Basolateral membrane vesicles were prepared from the ileum of male 180–200 g Sprague-Dawley rats according to the Percoll gradient separation method of Scalera et al. [25] as modified in our laboratory [30].

Enterocyte Preparation

Preparation of isolated, intact rat ileal cells was according to a modification of the method of Pinkus [24]. In brief, the small intestine was removed from three 180–200 g male Sprague-Dawley rats and divided into 10 equal segments. Segments 8–9 were flushed with warm (37°C), oxygenated, phosphate-buffered saline (PBS), at pH 7.4, consisting of (in mM) 137 NaCl, 8.16 Na_2HPO_4, 1.47 KH_2PO_4 and 3.22 KCl, and fortified with (in mM) 5.5 D-glucose and 2 L-glutamine. After clamping the distal end, the segments were filled with warm, oxygenated solution A, at pH 7.4, consisting of (in mM) 96 NaCl, 27 Na_3 citrate, 5.6 KH_2PO_4, 1.5 KCl, 5.5 D-glucose and 2 L-glutamine, and clamped proximally. The filled segments were incubated in oxygenated PBS at 37°C for 15 min in a shaker bath (75 osc/min). The intestinal segments were flushed and then filled with warm solution B consisting of oxygenated, fortified PBS, 1.5 mM EDTA, 1 mM dithiothreitol and 0.1% (wt/vol) BSA. The filled segments were incubated in oxygenated PBS at 37°C for 7.5 min in a shaker bath (100 osc/min). By gently squeezing the segments between the fingers, cells were collected in a plastic beaker containing ice-cold solution C consisting of (in mM) 122 NaCl, 25 $NaHCO_3$, 4.72 KCl, 2.56 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1 dithiothreitol and 1.0% BSA and gassed with 95% O_2/5% CO_2 to pH 7.4. The segments were flushed with solution C to remove remaining cells. The enterocytes were centrifuged at 400 × *g* in a refrigerated centrifuge, and the pelleted cells were washed in preincubation media selected for fractionation, transport or photolysis experiments. Cell viability was assessed by exclusion of 0.3% trypan blue dye. Only those preparations with >85% viable cells were used for study. In addition, oxygen consumption was recorded polarographically on random preparations with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Oxygen consumption rates (nmol O_2/min/mg protein) were 12.4 ± 5.5 (n = 7) and 18.3 ± 8.7 (n = 7), respectively, in the absence and presence of exogenous substrates (10 mM D-glucose, 5 mM L-glutamine). These values are similar to those reported by Pinkus [24].

Subcellular Fractionation

Ileal enterocytes were suspended in solution containing (in mM) 300 mannitol, 20 HEPES/Tris, at pH 7.4, and centrifuged at 400 × *g* for 5 min. Pelleted cells were suspended in ice-cold isolation buffer (3–5 mg protein/ml) consisting of 5% sorbitol, 0.5 mM EDTA, 5 mM histidine-imidazole, at pH 7.4, 0.05% soybean trypsin inhibitor, 0.25 mM phenylmethyl-sulfonylfluoride and 2 U/ml aprotinin. The suspended cells were disrupted in a Parr bomb (Parr Instrument Co., Moline, IL) under 800 psi at 0°C for one min/ml of sample. The cell homogenate was diluted to 3 times its original volume with isolation buffer and centrifuged at 7000 × *g* for 10 min. The top fluffy layer and supernatant were centrifuged at 200,000 × *g* for 60 min. The pellet was resuspended in isolation buffer using a 23-gauge needle and syringe. Sorbitol was added to make a final 50% sorbitol solution. A discontinuous sorbitol density gradient was made in the following order from the bottom layer: 1 ml of 60%, 1 ml of 55%, 3 ml pellet suspension in 50%, 2 ml of 40%, 2 ml of 30%, 2 ml of 20% and 1 ml of 10% sorbitol. The sorbitol gradient was centrifuged at 200,000 × *g* for 4 hours. Fractions were collected from the top of the gradient with an Auto-Densi-Flow system (Haake Buchler Instruments Inc., Saddle Brook, NJ). The basolateral membrane fraction was collected from the 30/40% sorbitol interface. The brush-border membrane fraction was collected from the 40/50% interface and the 50% sorbitol fraction and further purified following Mg^{2+} precipitation of heavy microsomes.

Enzyme and Protein Determinations

The purity of the subcellular fractions was assessed by determination of the activity of marker enzymes. K^+-stimulated *p*-nitrophenyl phosphatase was measured as described by Murer et al. [22]. Alkaline phosphatase (E.C.3.1.3.1) activity was measured according to the method of Weiser [31]. Galactosyltransferase activity was measured as described by Mahley et al. [20]. NADPH-cytochrome *c* reductase (E.C.1.6.2.4) was assayed by the method of Phillips and Langdon [23]. Protein was determined by the method of Lowry et al. [18] after precipitation with ice-cold 10% trichloroacetic acid using BSA as a standard.

Transport Measurements

Uptake of radiolabeled substrate by basolateral membrane vesicles was determined by the membrane filtration technique [8]. Typically, the transport reaction was initiated by adding 20 μl of preincubated membrane vesicles to 60–180 μl of incubation buffer kept in a water bath at 37°C. The compositions of the preincubation and incubation media are given in the legends to the figures. At desired time intervals, the transport reaction was terminated by the addition of 1 ml of ice-cold stop solution that had the same composition as the incubation medium plus 0.6 mM taurocholate, but no isotope. The entire contents were pipetted onto the middle of a prewashed, prechilled filter (cellulose nitrate, 0.45 μm pore size, Sartorius Filters, Inc., Hayward CA) kept under suction. The filter was rinsed immediately with 5 ml ice-cold stop solution and then solubilized in Brays solution. The

[1] The abbreviations used are: 7,7-azo-TC, (7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TC, taurocholate; PAH, para-amino-hippurate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EDTA, ethylenediamine-tetracetic acid; and BSA, bovine serum albumin.

radioactivity remaining on the filter was counted with standard liquid scintillation techniques. After correction of medium radioactivity bound to the filters in the absence of membrane vesicles, absolute solute uptake was calculated and is expressed as pmol/mg protein. All experiments were performed in triplicate with freshly prepared membrane vesicles on two or more separate days. Analysis of data for significant differences ($P < 0.05$) was according to the Student's t test for unpaired data [27].

For cell efflux experiments, 10 μl of ileal enterocytes were incubated for 4 min at 37°C in 90 μl of buffer containing [^{3}H] taurocholate to achieve steady-state conditions (i.e., uptake equals efflux). The efflux reaction was initiated by adding 2 ml of warm (37°C) efflux media. The compositions of the incubation and efflux media are provided in the figure legends. At desired time intervals, the efflux reaction was terminated by adding 200 μl aliquots of cell suspension to 1 ml of ice-cold stop solution (efflux media). The entire contents were filtered (cellulose nitrate filters, 0.65 μm pore size), washed and counted for radioactivity as described above for basolateral membrane vesicles.

Photoaffinity Labeling

Ileal basolateral membrane vesicles (400–500 μg of protein) were preloaded with buffer 1 consisting of (in mM) 150 tetramethylammonium, 50 potassium, 200 gluconate, 12 HEPES/Tris, at pH 7.4, plus 10 PAH. The preloaded membrane vesicles were incubated under red lighting at 25°C for 1 min in buffer 1 containing 40 μM [^{3}H]7,7-azo-TC. Photolysis was carried out with a total volume of 1.4 ml at 25°C for 10 min in a cylindrical cuvette made of Pyrex glass (Wilmad Glass Co., Buena, NJ) situated inside a Rayonet photoreactor RPR 100 (Southern New England Ultraviolet Co., Hamden, CT) equipped with 16 symmetrically arranged UV lamps. After photoaffinity labeling, the membranes were collected from the cuvette and washed 2 times by dilution in buffer 1 and centrifugation at 47,000 × g.

Ileal enterocytes were preincubated under red lighting at 25°C for 1 min in solution containing (in mM) 89.9 mannitol, 100 NaCl, 10 HEPES/Tris, at pH 7.4, and 0.025 [^{3}H] 7,7-azo-TC in a final concentration of 2.0–2.5 mg cell protein/ml. Photolysis was carried out for 10 min in the glass cuvette under conditions described above for ileal basolateral membrane vesicles. Following photoaffinity labeling, the cell suspension was removed from the cuvette, diluted in 30 ml of isolation buffer and centrifuged at 400 × g for 5 min. Separation of unbound radioactivity from the cells was achieved by this washing procedure ×2. After the second wash, the cells were suspended in isolation buffer for subcellular fractionation as described above.

Gel Electrophoresis

To achieve high resolution during SDS-PAGE, the radiolabeled samples were extracted 3 times with 700 μl of ethanol/chloroform solution (1 : 2 vol/vol). Separation of polypeptides from extracted lipids was achieved by centrifugation [12]. The extracted samples were dissolved in 100 μl of 62.5 mM Tris/HCl buffer, at pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromphenol blue by heating at 95°C for 5 min. SDS-PAGE was performed according to the procedure of Laemmli [17] with the total acrylamide concentration of 9% at a ratio of acrylamide : bisacrylamide of 97.2 : 2.8. The gels were fixed, stained with Coomassie brilliant blue R-250 and destained as previously described [12]. The stained polypeptides were detected by scanning the gels with a soft laser densitometer (LKB Instruments, Rockville, MD). The gels were then cut into slices of 2-mm thickness. Each slice was solubilized in a Protosol/water mixture (65 : 10, vol/vol). After solubilization overnight, 10 ml of Aquasol was added, and the samples were counted for radioactivity.

Materials

Transport and photoaffinity labeling were performed with the sodium salt of (7,7-azo-3α12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl-2-amino-ethanesulfonate having a specific radioactivity of 1.25 Ci/mmol. The synthesis and properties of this photolabile bile acid derivative have been previously described [14]. [^{3}H] taurocholate (6.8 Ci/mmol) and Protosol were purchased from New England Nuclear (Boston, MA). Taurocholate was purchased from Steraloids, Inc. (Wilton, NH). The radiopurity of the labeled bile acids was found to be greater than 95% using thin-layer chromatography.

Results

Marker Enzyme Activities

The isolation and separation of basolateral membranes from isolated ileal cells was carried out in two stages. After disruption of cells in the Parr bomb, the resulting organelles and membrane fragments were fractionated by differential centrifugation and sorbitol density gradient separation. Galactosyltransferase, K^+-stimulated p-nitrophenyl phosphatase, alkaline phosphatase and NADPH cytochrome c reductase were used as marker enzymes for Golgi, basolateral membranes, brush-border membranes and microsomes, respectively. Specific activities of these enzymes were measured in the differential centrifugation and density gradient fractions. The marker enzyme distribution of these fractions are summarized in the table. The relative enrichment of 10-fold over starting cell homogenate for p-nitrophenyl phosphatase in the 30–40% sorbitol fraction (F_2) identified this fraction as being relatively enriched in basolateral membranes. The specific activities of the Golgi and brush-border membrane enzymes were increased twofold compared to the homogenate, while that of the microsomal enzyme was unchanged. In F_2, the yields of galactosyltransferase, K^+-stimulated p-nitrophenyl phosphatase, alkaline phosphatase and NADPH-cytochrome c reductase were 21, 37, 12, and 6%, respectively. The total recovery for each of these enzymes in all fractions was at least 80% of the activity of the cell homogenate. In comparison, alkaline phosphatase was the only enzyme that was

Table. Marker enzyme specific activities of the fractions obtained from rat ileal cells

Fraction	Galactosyl-transferase (cpm × 10^{-3}/mg/hr)		K^+-stimulated *p*-nitrophenyl phosphatase (nmol/mg/min)		Alkaline phosphatase (nmol/mg/min)		NADPH-Cyt.C Red. (nmol/mg/min)	
	(*E*)[a]	%Y[b]	(*E*)	%Y	(*E*)	%Y	(*E*)	%Y
Homogenate	1.33 ± 0.24[c] (1)		143 ± 6.7 (1)		400 ± 34.1 (1)		45.3 ± 4.0 (1)	
F I(20/30)[d]	16.29 ± 2.22 (12.2)	27	180 ± 24.8 (1.26)	4	199 ± 70.2 (0.49)	—	16.5 ± 3.7 (0.36)	—
F II(30/40)	2.71 ± 0.54 (2.04)	21	1435 ± 165.0 (10.03)	37	811 ± 9.4 (2.03)	12	50.3 ± 10.1 (1.11)	6
F III(40/50) + 50)	2.06 ± 0.21 (1.55)	11	146 ± 51.6 (1.02)	8	1874 ± 63.1 (4.68)	37	39.3 ± 19.5 (0.87)	20
F IV(40/50 + 50)	1.45 ± 0.37 (1.09)	9	180 ± 77.2 (1.26)	7	1188 ± 168.0 (2.97)	10	190.1 ± 40.2 (4.20)	35
F V(55/60)	0.95 ± 0.34 (0.71)	5	184 ± 66.5 (1.29)	3	567 ± 126.0 (1.42)	7	99.3 ± 5.7 (2.19)	9
F VI(pellet)	—	1	382 ± 1.4 (2.67)	13	562 ± 146.0 (1.40)	5	160.5 ± 8.3 (3.54)	11

[a] Enrichment over homogenate.
[b] Percentage yield; values represent the mean of 3 experiments.
[c] Values represent the mean ± SE for 3 experiments in duplicate determinations.
[d] Values in parentheses represent % of sorbitol for the different fractions.

relatively enriched (4.7-fold) in F_3 indicating that this fraction was enriched in brush-border membranes.

Interaction of 7,7-azo-TC with the Anion Exchange Transport System in Ileal Basolateral Membrane Vesicles under Red Lighting

In order to identify putative transport proteins by photoaffinity labeling, it first must be demonstrated that the photolabile derivative can share the transport system with natural substrate [37]. Previous studies showed that the imposition of an intravesicular to extravesicular anion gradient resulted in the stimulation of taurocholate uptake by ileal basolateral membrane vesicles [30]. Figure 1*A* shows that the preloading of ileal basolateral membrane vesicles with the organic anion PAH resulted in the stimulation of [^{3}H] taurocholate uptake with respect to unpreloaded vesicles. In addition, increasing concentrations of unreacted 7,7-azo-TC under red lighting progressively inhibited PAH-transstimulated taurocholate uptake but had no effect on taurocholate uptake in the absence of PAH. At 100 μM 7,7-azo-TC, the PAH-*trans*stimulated component of taurocholate uptake (i.e., uptake in the presence of a PAH gradient minus uptake in the absence of PAH) was completely abolished. Conversely, Fig. 1*B* shows that increasing concentrations of taurocholate progressively inhibited PAH-*trans*-stimulated [^{3}H] 7,7-azo-TC uptake but had no effect on the uptake of the unreacted photolabile derivative in the absence of PAH. PAH-*trans*stimulated 7,7-azo-TC uptake was nearly abolished at 100 μM taurocholate. These observations suggest that 7,7-azo-TC shared the anion exchange transport system with the natural bile acid.

Inhibition of Taurocholate Uptake in Ileal Basolateral Membrane Vesicles by Photoaffinity Labeling

To test whether the inhibition of taurocholate uptake by 7,7-azo-TC is reversible without photolysis, ileal basolateral membrane vesicles were preincubated without or with 100 μM 7,7-azo-TC under red lighting. After washing the vesicles in medium free of 7,7-azo-TC, the uptake of 100 μM [^{3}H] taurocholate subsequently was measured in the presence and absence of an externally directed PAH gradient. As shown in Fig. 2 (bars 1 and 2), no significant difference in taurocholate uptake was seen between vesi-

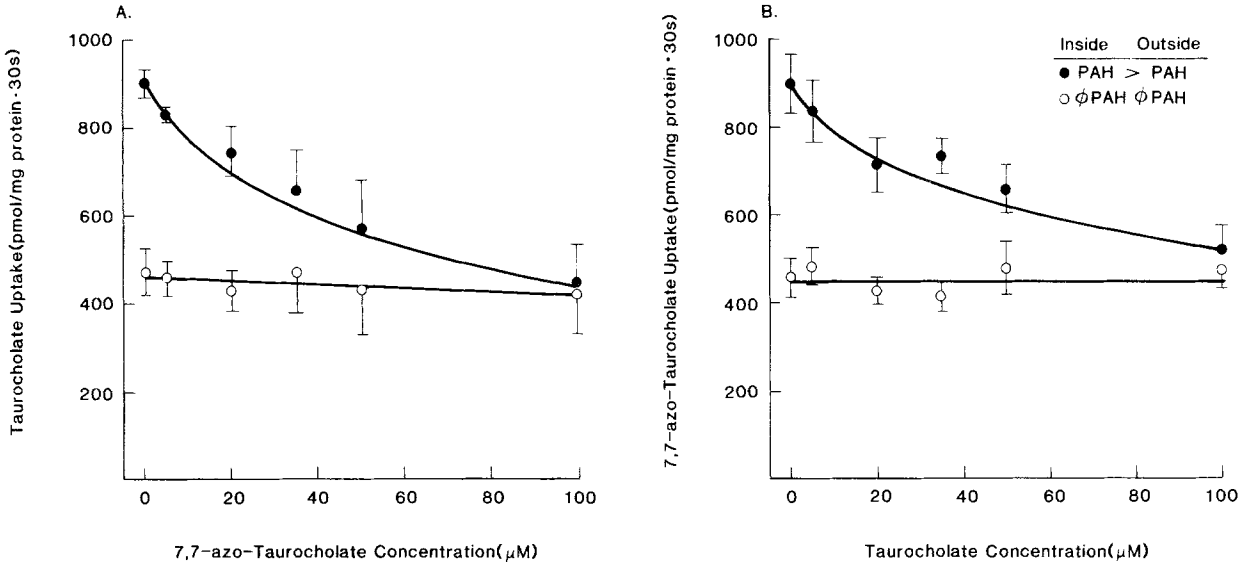

Fig. 1. Effect of 7,7-azo-TC on taurocholate (*A*) and taurocholate on 7,7-azo-TC (*B*) uptake by ileal basolateral membrane vesicles. Vesicles were preloaded with 150 mM tetramethylammonium, 50 mM potassium, 200 mM gluconate, 12 mM HEPES/Tris buffer, at pH 7.4, and 0 (○) or 10 mM PAH and 190 mM gluconate (●), and then uptake of 100 μM [^{3}H] taurocholate (*A*) or 100 μM [^{3}H]7,7-azo-TC (*B*) was assayed in the same medium containing 0 mM PAH and the indicated concentrations of 7,7-azo-TC (*A*) or taurocholate (*B*). All experiments were performed in the presence of valinomycin and FCCP to minimize indirect effects on transport that arise from transmembrane pH and potential differences generated by test anions. The experiments were performed under red lighting to prevent photolysis of 7,7-azo-TC. Means ± SE of nine determinations (three preparations) are shown for *A* and 6 determinations (two preparations) for *B*

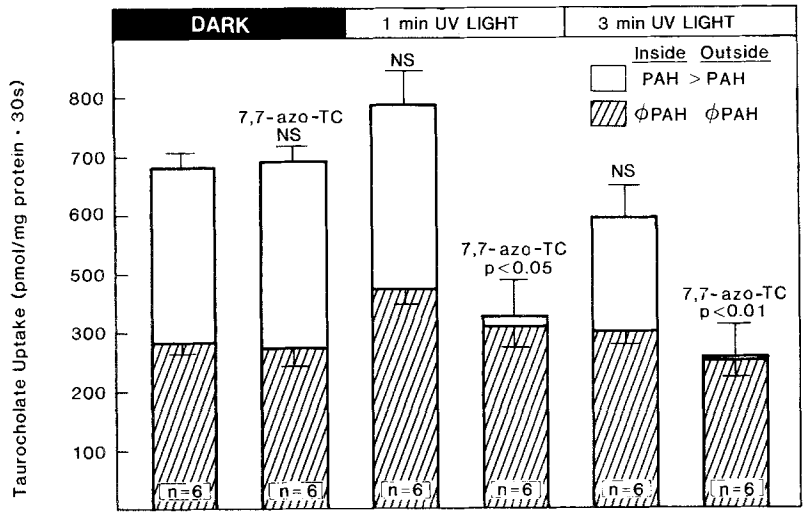

Fig. 2. Effect of pretreatment with 7,7-azo-TC and irradiation on taurocholate uptake by ileal basolateral membrane vesicles. Vesicles preloaded with 0 (hatched bars) or 10 (open bars) mM PAH were incubated in the absence (bars 1, 3, 5) or presence (bars 2, 4, 6) of 100 μM 7,7-azo-TC and irradiated for 0 (bars 1, 2), 1 (bars 3, 4) or 3 (bars 5, 6) min. After washing out removable 7,7-azo-TC, uptake of 100 μM [^{3}H] TC was assayed in the same medium containing 0 mM PAH under red lighting. The composition of the buffers is provided in the legend to Fig. 1*A*. The means are shown ± SE for the number (*n*) of determinations. Nonsignificant (*NS*) and significant differences are indicated

cles preincubated without or with the photolabile derivative. Since these observations indicated that the inhibition by 7,7-azo-TC under red lighting was fully reversible, the next series of experiments was performed to determine whether photolysis of 7,7-azo-TC would result in an irreversible inhibition of bile acid transport. Irradiation of the vesicles for 1 min in the absence of 7,7-azo-TC (Fig. 2, bar 3) had no effect on subsequent transport of taurocholate when compared to nonirradiated control vesicles (Fig. 2, bar 1). However, if the ileal basolateral membrane vesicles were exposed to UV light for 1

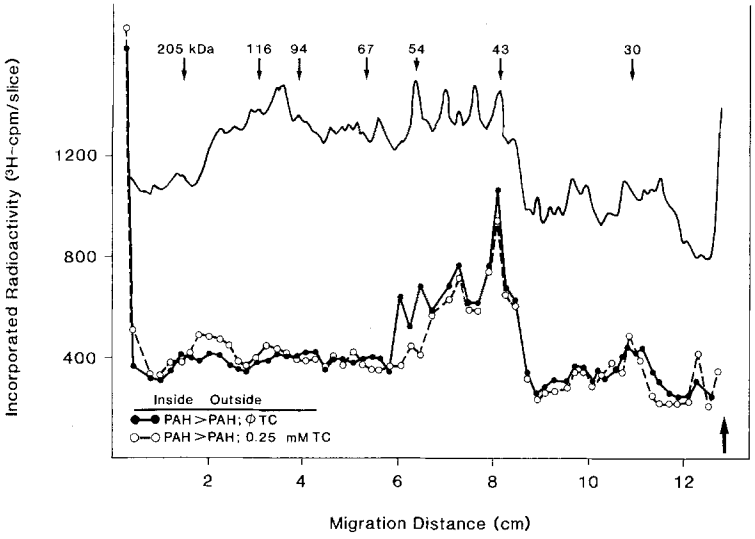

Fig. 3. Distribution of radioactivity after photoaffinity labeling with [^{3}H] 7,7-azo-TC and SDS-PAGE of ileal basolateral membrane vesicles. Membrane vesicles that were preloaded with 10 mM PAH, were photolabeled with 40 μM (30 μCi) [^{3}H] 7,7-azo-TC in the presence of 0 (●—●) or (O--O) 0.25 mM TC. The arrow shows the position of bromphenol blue. The top solid line shows the densitometer scanning of the polypeptides after staining, whereas the bottom circled lines indicate the distribution of radioactivity

min in the presence of 100 μM 7,7-azo-TC, PAH-*trans*stimulated taurocholate uptake was found to be almost completely inhibited (Fig. 2, bar 4). When membranes were exposed to UV light for 3 min in the absence of 7,7-azo-TC, subsequent taurocholate uptake was not significantly reduced (Fig. 2, bar 5). However, 3 min irradiation in the presence of the photolabile derivative resulted in complete inhibition of PAH-*trans*stimulated taurocholate uptake (Fig. 2, bar 6). Photoaffinity labeling under all conditions had no effect on taurocholate uptake in the absence of PAH.

Photoaffinity Labeling of Ileal Basolateral Membrane Vesicles

For these studies, ileal basolateral membrane vesicles preincubated in the presence of PAH were incubated in media containing [^{3}H]7,7-azo-TC and exposed to UV light. The photolyzed membrane vesicles were washed and subjected to SDS-PAGE. As shown in Fig. 3, photoaffinity labeling of membrane vesicles resulted in an incorporation of radioactivity into several polypeptides. The apparent molecular weights of the clearly labeled polypeptides were 43,000, 47,000, 54,000 and 59,000. Repeated experiments revealed that this labeling pattern was highly reproducible even in the absence of PAH. Moreover, to gain further evidence that the photolabile derivative is bound to the same polypeptide as natural bile acid, photoaffinity labeling of ileal basolateral membrane vesicles was performed in the presence of 0.25 mM taurocholate. Figure 3 demonstrates that taurocholate preferentially inhibited the labeling of the 54,000 and 59,000 mol. wt. polypeptides.

Interaction of Unreacted 7,7-azo-TC and PAH with Bile Acid Efflux from Ileal Enterocytes

In order to determine which putative transport proteins are photolabeled when the cellular structures are preserved, studies were performed with enterocytes freshly prepared from rat ileum. To reflect transport events at the basolateral membrane, the efflux of taurocholate from ileal cells was determined. Initial studies were performed to demonstrate that 7,7-azo-TC can share this transport system with the natural bile acid. As shown in Fig. 4*A*, ileal cells achieved similar intracellular levels of taurocholate (i.e., pmol/mg protein) following preincubation with 20 μM [^{3}H] taurocholate in the presence or absence of 50 μM 7,7-azo-TC. When the preloaded cells were added to efflux media free of bile acid, 7,7-azo-TC resulted in a significant inhibition of taurocholate efflux from cells (Fig. 4*A*). Inasmuch as taurocholate uptake by ileal basolateral membrane vesicles is *trans*stimulated by PAH (Fig. 1*A*), the efflux rate of taurocholate was determined with or without PAH in the efflux media. Figure 4*B* shows that the addition of 10 mM PAH to the efflux media significantly enhanced the efflux rate of taurocholate from ileal cells preincubated with 20 μM [^{3}H] taurocholate. These data suggest that the efflux of taurocholate from ileal enterocytes is *cis*-inhibited by 7,7-azo-TC and *trans*stimulated by PAH.

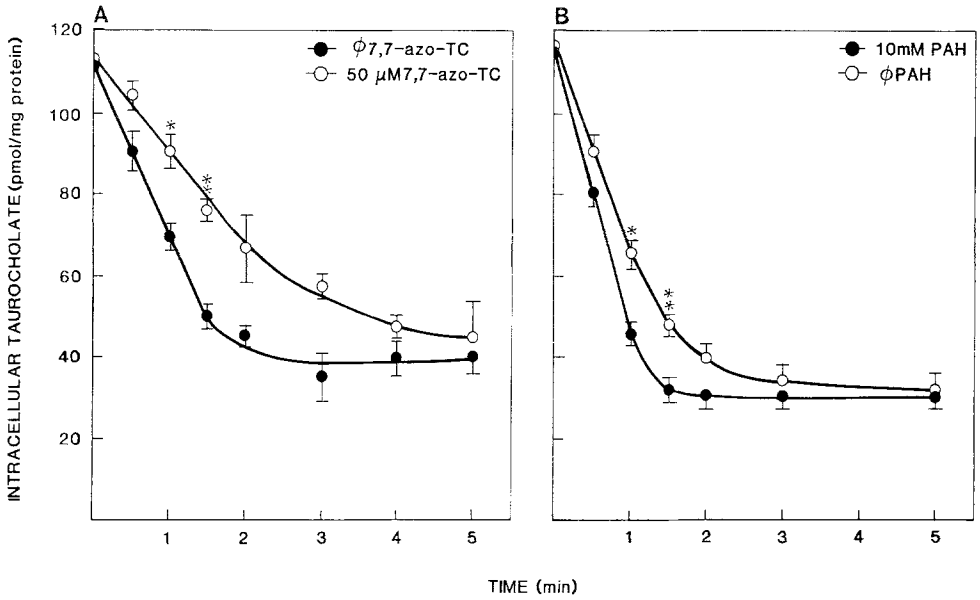

Fig. 4. The effect of 7,7-azo-TC *cis*-inhibition (*A*) and PAH *trans*-stimulation (*B*) on taurocholate efflux from ileal enterocytes. (*A*): Ileal cells were incubated with buffer containing 122 mM NaCl, 25 mM $NaHCO_3$, 4.72 mM KCl, 2.56 mM $MgCl_2$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, at pH 7.4, 0.02 mM [^{3}H] taurocholate, and 0 (—●—) or 0.05 (—○—) 7,7-azo-TC. The cells were then exposed to the same buffer free of bile acids with choline Cl substituted for NaCl (efflux buffer). (*B*): Ileal cells were incubated with the same buffer as *A*, free of 7,7-azo-TC and then exposed to efflux buffer containing 270 mM mannitol, 10 mM HEPES/Tris, at pH 7.4, and 0 (—●—) or 10 (—○—) mM PAH. Each point is the mean ± SE for six determinations. * = $P < 0.05$; ** = $P < 0.001$

Inhibition of Taurocholate Efflux from Ileal Enterocytes by Photoaffinity Labeling

Figure 5 shows the effect of irradiation and pretreatment with 7,7-azo-TC on the subsequent efflux of taurocholate by ileal enterocytes. Ileal cells were preincubated with 20 μM [^{3}H] taurocholate in the absence or presence of 25 μM 7,7-azo-TC under red lighting. After adding the cells to efflux media free of bile acid, the efflux of taurocholate was measured by subtracting cellular taurocholate concentration at 90 sec from cellular concentration at 0 sec. As shown in Fig. 5 (bars 1 and 2), a significant decrease in taurocholate efflux was seen from cells preincubated in the presence of the photolabile derivative. Figure 5 (bar 3) shows that irradiation of cells for 5 min in the absence of 7,7-azo-TC had no effect on the subsequent efflux of taurocholate when compared to nonirradiated control cells (Fig. 5, bar 1). Thus, UV light, by itself, had no adverse effect on efflux activity. In contrast, after irradiation of cells in the presence of 7,7-azo-TC (Fig. 5, bar 4), taurocholate efflux was found to be inhibited to a significantly greater degree ($P < 0.01$) than preincubation in the presence of the photolabile derivative under red lighting (Fig. 5, bar 2).

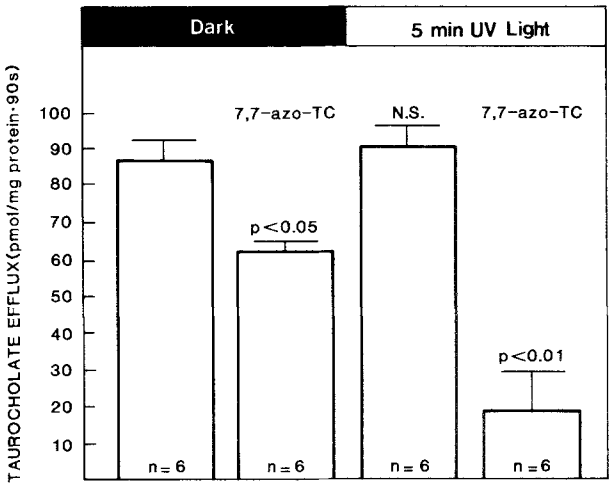

Fig. 5. Effect of pretreatment with 7,7-azo-TC and irradiation on taurocholate efflux by ileal enterocytes. Ileal cells preloaded with 0.02 mM [^{3}H] taurocholate were incubated in the absence (bars 1, 3) and presence (bars 2, 4) of 0.025 mM 7,7-azo-TC and irradiated for 0 (bars 1, 2) or 5 (bars 3,4) min. The cells then were exposed to the same buffer (free of bile acid), and the efflux of [^{3}H] taurocholate was assayed under red lighting. The compositions of the incubation and efflux buffers are provided in the legend to Fig. 4*A*. The means are shown ± SE for the number (*n*) of determinations. Nonsignificant (*N.S.*) and significant differences are indicated

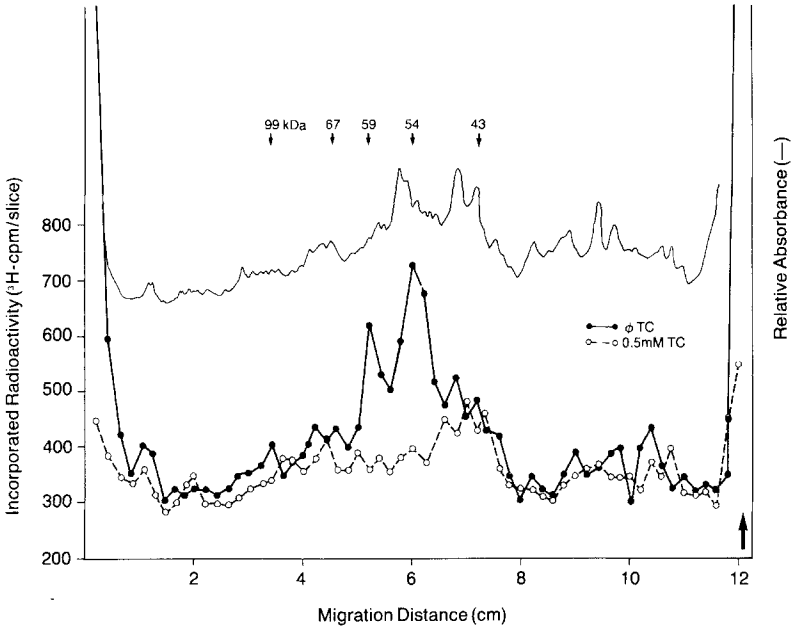

Fig. 6. Distribution of radioactivity after SDS-PAGE of basolateral membrane fration isolated from ileal cells irradiated with [^{3}H]7,7-azo-TC. Cell suspensions were preincubated for 1 min with 25 μM (75 μCi) [^{3}H]7,7-azo-TC and irradiated at 25°C for 10 min. Cells then were resuspended in isolation buffer, washed 2 times, disrupted and fractionated. The basolateral membrane-enriched fraction was subjected to SDS-PAGE. The arrow indicates the position of bromphenol blue. The top solid line shows the densitometer scanning of the polypeptides after staining, whereas the bottom circled lines indicate the distribution of radioactivity following photolysis of cells in the absence (●—●) or presence (O--O) or 0.5 mM TC

Photoaffinity Labeling of Ileal Enterocytes

Photoaffinity labeling of intact cells followed by cell fractionation allows for the detection of bile acid-binding polypeptides in the relatively native, unperturbed state. For these studies, ileal enterocytes were photolyzed in the presence of 25 μM [^{3}H]7,7-azo-TC and then subjected to subcellular fractionation. The fraction containing the highest enrichment of the basolateral membrane marker, Na^+-K^+-ATPase activity, was subjected to SDS-PAGE. Figure 6 shows the photoaffinity labeling pattern of the basolateral membrane fraction (F_2) from ileal cells irradiated with [^{3}H]7,7-azo-TC. As shown in Fig. 6 (solid line with filled circles), greatest incorporation of radioactivity was seen in polypeptides of 54,000 and 59,000 mol. wt. In order to gain further evidence that the photolabile derivative was bound to the same polypeptide as natural bile acid, photoaffinity labeling of ileal enterocytes was performed in the presence of 0.5 mM taurocholate. As shown in Fig. 6 (broken line with open circles), taurocholate selectively inhibited photoaffinity labeling of the 54,000 and 59,000 mol. wt. polypeptides.

The labeling of 54,000 and 59,000 mol. wt. polypeptides had not been observed in previous studies with ileal brush-border membrane vesicles, whereas 43,000 and 99,000 mol. wt. polypeptides were clearly labeled [12]. For purposes of comparison, the subcellular fraction containing the highest enrichment of the brush-border membrane marker was subjected to SDS-PAGE. Figure 7 shows the pattern of incorporation of radioactivity into polypeptides of the brush-border membrane fraction (F_3) that was obtained from ileal cells irradiated with [^{3}H]7,7-azo-TC. As shown in Fig. 7 (solid line with filled circles), greatest incorporation of radioactivity was seen in polypeptides of 43,000 and 99,000 mol. wt. Figure 7 (broken line with open circles), shows that taurocholate inhibited the photoaffinity labeling of these polypeptides.

Discussion

Although the uptake of bile acids by the ileal brush-border membrane has been well-characterized as a Na^+-dependent, secondary active transport system, relatively little information has been obtained regarding the mechanisms responsible for the exit of bile acids from the enterocyte. Once bile acids reach the basolateral membrane, it is possible that their movement from the cell is directed downward from a region of higher concentration to one of lower concentration despite the likelihood that binding lowers free intracellular activity. Furthermore, the movement of bile acid anion out of the cell is favored by the intracellular negative potential [33]. Thus, the transport of bile acid across the basolateral membrane may not require a facilitated carrier-mediated transport system. However, recent studies with basolateral membrane vesicles demonstrated that bile acid transport could be *trans*stimulated and *cis*-inhibited by a variety of organic and inorganic anions [30]. These observations

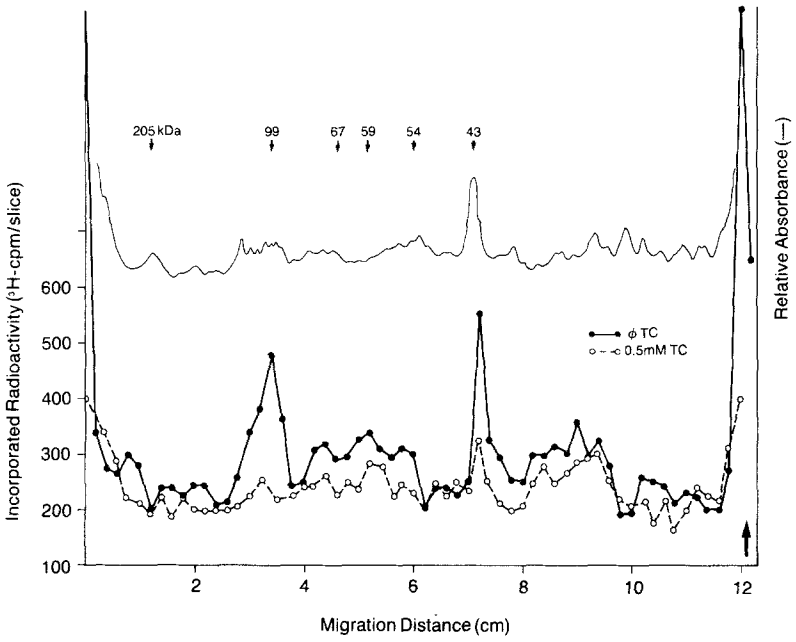

Fig. 7. Distribution of radioactivity after SDS-PAGE of brush-border membrane fraction isolated from ileal cells irradiated with [^{3}H]7,7-azo-TC. Cells were irradiated and fractionated as described in the legend to Fig. 6. The brush-border membrane-enriched fraction was subjected to SDS-PAGE. The top solid line indicates the densitometer scanning of the polypeptides after staining, whereas the bottom circled lines indicate the distribution of radioactivity following photolysis of cells in the absence (●—●) or presence (○--○) of 0.5 mM TC

suggested that bile acids could be transported by anion exchange and that different transporters exist for bile acids in the brush-border and basolateral membranes of the ileal epithelial cell.

An approach to the identification of membrane proteins involved in the bile acid transport systems is photoaffinity labeling. Photolabile derivatives of bile acids have been used successfully to identify bile acid-binding polypeptides in blood [1, 13], hepatocytes [6, 28, 29], liver plasma membranes [1, 10, 11, 32], and brush-border membranes of intestine [12] and kidney [4]. In order to detect putative transport proteins, the present studies involved the photolysis of ileal basolateral membrane vesicles in the presence of a photolabile bile acid derivative followed by SDS-PAGE. Initial studies demonstrated that the photolabile derivative had transport characteristics like those of the natural substrate and was able to interact with the transport system. As shown previously for taurocholate [30], the establishment of an externally directed anion gradient with PAH stimulated the uptake of 7,7-azo-TC in ileal basolateral membrane vesicles. The PAH-*trans*stimulated uptake of 7,7-azo-TC was inhibited by taurocholate and vice versa. The inhibition of taurocholate uptake by unreacted 7,7-azo-TC was completely reversible, i.e., taurocholate uptake was completely restored following the incubation of membrane vesicles with 7,7-azo-TC and subsequent washing under red lighting. Photolysis, by itself, had no effect on subsequent taurocholate uptake, whereas photolysis in the presence of 7,7-azo-TC inhibited irreversibly PAH-driven uptake of taurocholate in ileal basolateral membrane vesicles. This irreversible inhibition indicated covalent modification of membrane proteins involved in bile acid transport. Thus, 7,7-azo-TC appeared well-suited for the identification of bile acid-binding polypeptides. When ileal basolateral membrane vesicles were photolyzed in the presence of [^{3}H]7,7-azo-TC and then subjected to SDS-PAGE, several polypeptides were radiolabeled. Selective inhibition of radioactive incorporation by taurocholate suggested that the photolabile derivative competed with natural bile acid for the binding to 54,000 and 59,000 mol. wt. polypeptides.

The present studies also involved the photolysis of isolated ileal enterocytes in the presence of a photolabile bile acid derivative followed by subcellular fractionation and SDS-PAGE. This approach has the advantage in that it allows for the accessibility to the photoprobe of only those membrane components that are normally available to bile acid in the intact cell. The directional polarity of bile acid transport in ileal enterocytes suggests that efflux from preloaded cells reflects transport events across the basolateral membrane [35]. A disadvantage of this approach with intact cells is that reversible and irreversible inhibition of transport by a photolabile derivative cannot be demonstrated directly as with plasma membrane vesicle systems. However, present studies indicated that 7,7-azo-TC *cis*-inhibited taurocholate efflux from enterocytes under red lighting. Moreover, photolysis of preloaded cells in the presence of 7,7-azo-TC inhibited taurocholate efflux to a greater extent than in the presence of the photolabile derivative under subdued lighting. This greater inhibition suggested an

irreversible biochemical modification of the bile acid transport system by the photoactivated derivative. When photolyzed ileal cells were fractionated and the resultant basolateral membrane-enriched fraction was subjected to SDS-PAGE, radioactivity from [^{3}H] 7,7-azo-TC was incorporated into polypeptides of the same molecular weight as identified by direct photoaffinity labeling of basolateral membrane vesicles.

Another advantage of photoaffinity labeling of intact cells is that bile acid-binding polypeptides can be compared between different subcellular fractions. Previous photoaffinity labeling studies identified bile acid-binding polypeptides of 43,000, 67,000, 83,000, 99,000 and 125,000 mol. wt. in brush-border membrane vesicles from rat ileum [12]. The 43,000 and 99,000 mol. wt. polypeptides were most clearly and consistently labeled with the photolabile bile acid derivatives. A 94,000 mol. wt. polypeptide, but not the 99,000 mol. wt. polypeptide, was labeled in jejunal brush-border membrane vesicles [12]. The 43,000 and 67,000 mol. wt. polypeptides were identified as actin [6] and albumin [6, 11], respectively. On the basis of these observations, it was speculated that the 99,000 mol. wt. polypeptide is involved in Na^+-dependent bile acid transport, which is restricted to the ileum [12]. In the present studies, the labeling of a 99,000 mol. wt. polypeptide from the brush-border membrane fraction that was obtained from isolated ileal cells, which were photolyzed in the presence of 7,7-azo-TC, reaffirms this speculation.

The ileum, kidney and liver are provided with specific transport systems for bile acids. Therefore, it is of interest to know whether homologies exist between bile acid-binding proteins in plasma membranes from these tissues. Recent photoaffinity labeling studies have associated a Na^+-dependent bile acid transport system with the identification of a 99,000 mol. wt. bile acid-binding polypeptide in brush-border membrane vesicles from the proximal tubule of rat kidney [4]. A number of affinity and photoaffinity labeling studies [1, 6, 10, 11, 28, 29, 32, 38–41] with hepatocytes and enriched liver plasma membranes have identified several bile acid-binding polypeptides. Whether these putative hepatic transport proteins are identical to the intestinal polypeptides described in the present report awaits further investigation.

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