

# Extrahepatic Obstructive Cholestasis Reverses the Bile Salt Secretory Polarity of Rat Hepatocytes

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## Abstract

To elucidate the consequences of extrahepatic cholestasis on the structure and function of hepatocytes, we studied the effects of bile duct ligation on the turnover, surface distribution, and functional activity of the canalicular 100-kD bile salt transport protein (cBSTP). Basolateral (bLPM) and canalicular (cLPM) liver plasma membrane vesicles were purified to the same degree from normal and cholestatic rat livers and the membrane bound cBSTP identified and quantitated using polyclonal anti-cBSTP antibodies. Cholestasis of 50 h resulted in an increased release of cBSTP into bile, thereby decreasing its *in vivo* half-life from 65 to 25 h. Furthermore, a significant portion of cBSTP accumulated at the basolateral surface and in intracellular vesicles of cholestatic hepatocytes. This redistribution of cBSTP was functionally paralleled by decreased and increased electrogenic taurocholate anion transport in cLPM and bLPM vesicles, respectively. These results demonstrate that biliary obstruction causes a reversal of the bile salt secretory polarity of rat hepatocytes. The resulting increase in basolateral (sinusoidal) bile salt efflux might protect hepatocytes from too high an accumulation of toxic bile salts within the cell interior.

## Introduction

Mechanical obstruction of extrahepatic bile ducts results in increased biliary pressure, failure of canalicular bile flow on the level of hepatocytes, and accumulation of obligatory cholephilic compounds such as bile salts, bilirubin, and cholesterol within liver cells and blood plasma. In hepatocytes the most prominent pathologic alterations are initially seen at the excretory pole and include dilatation of bile canaliculi, reduction and loss of canalicular microvilli, and disorganization and increased permeability of the tight junctional complexes (1–4). However, using experimental bile duct ligation, an unambiguous animal model for extrahepatic mechanical obstruction in humans, numerous studies have shown that obstructive chole-

stasis affects many other parts of liver cells as well. For example, the amounts of Golgi complex and of smooth and rough endoplasmic reticulum are decreased while the number of peroxisomes, pericanalicular vesicles, and lysosomes as well as the thickness of the pericanalicular microfilamentous web are increased (2, 3, 5). Especially intriguing is the observation of retrograde vesicle-mediated transport from bile to blood for lipoprotein X (6) and for dimeric IgA (7), suggesting that retrograde passage via the transcellular route might represent an important pathway for “regurgitation” of bile constituents into blood plasma. Such retrograde diacytosis might also be involved, at least in part, in the described topographic shift of some histochemically demonstrable enzymes (8–10) and antigens (11) from the canalicular to the basolateral (sinusoidal and lateral) membrane domain. Although these findings have been interpreted to reflect a “reversed secretory polarity” in cholestatic hepatocytes (2, 7), direct structural and functional proof for this hypothesis has so far not been obtained. Therefore, we investigated the turnover, surface distribution, and functional activity of the recently characterized canalicular bile salt transport protein (cBSTP)<sup>1</sup> in intact liver and in basolateral (bLPM) and canalicular (cLPM) liver plasma membrane (LPM) vesicles isolated from normal and bile duct-ligated rats (12–16). The results demonstrate an increased release of cBSTP into bile of obstructed animals. Furthermore, a significant portion of functionally active cBSTP accumulated within the basolateral membrane of cholestatic hepatocytes, thus providing direct evidence for reversal of the bile salt secretory polarity of hepatocytes during obstructive cholestasis.

## Methods

**Materials.** L-[<sup>35</sup>S]Methionine (1,060 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). [6-<sup>3</sup>H]-Taurocholate (6.6 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). Protein A-Sepharose CL-4B was from Pharmacia-Schweiz AG (Dübendorf, Switzerland). Nitrocellulose sheets, horseradish peroxidase-conjugated goat anti-rabbit IgG and the horseradish peroxidase color development reagent containing 4-chloro-1-naphthol and Bradford color-reagent were from Bio-Rad Laboratories AG (Glattbrugg, Switzerland). Newborn calf serum was from Sera-Lab (Sussex, England). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

**Animals.** Male Sprague-Dawley rats (SUT:STD, Süddeutsches Tierzuchtinstitut, Tuttlingen, Federal Republic of Germany) weighing 200–250 g were used throughout this study. The animals had free access to water, were fed *ad lib.* (na. 690 diet, Nafag, Gossau, Switzerland) and housed in a constant temperature and humidity environ-

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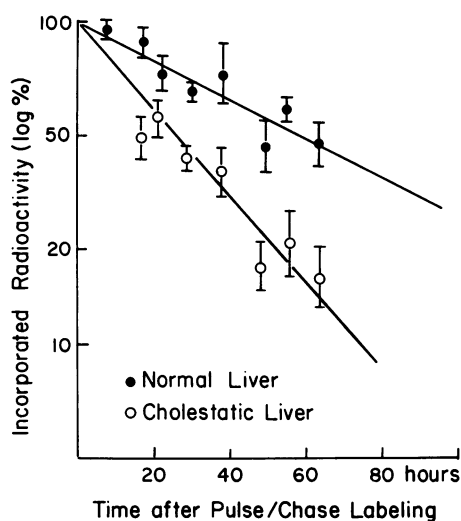
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1. *Abbreviations used in this paper:* bLPM, basolateral liver plasma membranes; cLPM, canalicular liver plasma membranes; cBSTP, canalicular bile salt transport protein; RE, relative enrichment; LPM, liver plasma membranes.

ment with alternating 12-h light (6:30 a.m. to 6:30 p.m.) and dark cycles.

Extrahepatic obstruction was produced by bile duct ligation under pentobarbital (sodium salt, 50 mg/kg intraperitoneally) anesthesia. The common bile duct was ligated close to the liver hilus to prevent damage to pancreatic ducts. Sham-operated animals were handled in a similar manner without ligation of the bile duct. Both groups of animals were allowed to eat ad lib. after surgery. After various postoperative periods (maximal 72 h) rats were regularly killed by cervical dislocation at 8:00 a.m.

**Isolation of LPM subfractions.** Essentially the same procedure was used for isolation of mixed LPM, bLPM, and cLPM subfractions as previously described (17). However, the reported high-yield, zonal centrifugation procedure (17) had to be scaled down and adapted to one to three rat livers in order to permit the parallel isolation of LPM subfractions from sham-operated and bile duct-ligated animals at the same day. In addition, the simultaneous preparation of mixed LPM from up to six livers was necessary for determination of the half-life of the 100-kD bile salt transport polypeptide (Fig. 1). Therefore, 10–30-g liver portions from differently treated animals were separately homogenized in 1 mM NaHCO<sub>3</sub>, pH 7.4, as described (17). After centrifugation at 1,500 *g*<sub>ave</sub> for 15 min (GSA rotor, Du Pont Instruments, Sorvall Biomedical Div., Newtown, CT), each crude nuclear pellet originating from one rat liver (~10 g) was resuspended in 2.2 vol of 70% sucrose (wt/wt, *d* 1.2623) to a final volume of ~40 ml. The suspensions were stirred on ice for 10 min to disrupt membrane aggregations. 15-ml samples of each diluted crude nuclear suspension were then overlaid with 12 ml of 44% sucrose (wt/wt, *d* 1.1972) followed by 10 ml of 36.5% sucrose (wt/wt, *d* 1.1587), and the centrifuge tubes (Ultra-Clear, 25 × 89 mm, Beckman Instruments, Inc., Palo Alto, CA) were filled to



**Figure 1.** Degradation half-life of membrane bound cBSTP in normal and cholestatic rat liver. Rats were pulse-labeled with L-[<sup>35</sup>S]-methionine (0.5 mCi intraperitoneally) and chases of unlabeled methionine (50 mg intraperitoneally) administered after 2 h and subsequently at 12-h intervals. 12 h after pulse-labeling the animals were sham-operated (●) or subjected to bile duct ligation (○). After the indicated time intervals (zero time = pulse-labeling), mixed LPM fractions were isolated and membrane-bound cBSTP quantitatively immunoprecipitated from solubilized LPM extracts. The radioactivity in immunoprecipitates was determined by liquid scintillation counting. Three animals corresponding to different postoperative periods were processed in parallel with the appropriate controls at the same day. Each illustrated time point represents the means ± SD of three independent experiments. Individual points were fitted by linear regression analysis and the lines extrapolated back to 100% incorporated radioactivity. Note the logarithmic scale of the ordinate.

the top with 0.25 M sucrose. The discontinuous sucrose gradients were centrifuged at 95,000 *g*<sub>ave</sub> for 150 min (AH 627 swing-out rotor, Du Pont Instruments, Sorvall Biomedical Div.). After centrifugation mixed LPMs were recovered from the 36.5%/44% sucrose interface, washed twice in 40 ml of 1 mM NaHCO<sub>3</sub>, and finally collected at 2,700 *g*<sub>ave</sub> for 15 min. Subsequent separation of bLPM and cLPM subfractions from the mixed LPM was exactly as described (17). This modified isolation procedure permitted to individually process up to six different livers (10 g each) at the same day as required for the half-life studies described below. The isolated LPM subfractions were resuspended in the appropriate buffer media and aliquots were stored frozen in liquid nitrogen (protein concentration > 5 mg/ml) for up to 2 wk without loss of transport functions. Protein was determined by the method of Bradford (18) using bovine  $\gamma$ -globulin as standard.

**Enzymatic characterization of isolated LPM subfractions.** Frozen samples were quick-thawed immediately before use by immersion of the tubes into a 37°C waterbath. The ouabain-sensitive Na<sup>+</sup>K<sup>+</sup> ATPase and the Mg<sup>2+</sup>-ATPase activities were determined by the coupled kinetic assay as modified by Scharschmidt et al. (19). Leucineamino-peptidase activity was measured according to Goldberg and Rutenburg (20). Alkaline phosphatase activity was assayed as described (21) using *p*-nitrophenylphosphate as substrate.  $\gamma$ -Glutamyltranspeptidase activity was determined according to Orłowski and Meister (22). To test for the presence of intracellular organelles, we measured the following marker enzyme activities: succinate cytochrome *c* reductase for mitochondria (23), NADPH cytochrome *c* reductase for microsomes (23), acid phosphatase for lysosomes (24), and galactosyltransferase for Golgi membranes (25).

**SDS gel electrophoresis.** SDS-PAGE was performed as described (26) using a Mini PROTEAN II dual-slab cell (Bio-Rad Laboratories AG) and a polyacrylamide concentration of 9.5%. The separated polypeptide bands were stained either with Coomassie brilliant blue R 250 (27) or alternatively by the silver stain technique (28).

**Antibody preparation against the 100-kD cBSTP.** The same polyclonal rabbit antibodies as previously characterized (15) were used throughout this study.

**Immunoblotting and immunoprecipitation procedures.** cBSTP was selectively detected in and immunoprecipitated from solubilized LPM subfractions as described (15). In all immunoprecipitation assays quantitative recovery of cBSTP was assessed by immunoblotting of the corresponding supernatants.

**Determination of the half-life of membrane-bound cBSTP in vivo.** Rats were pulse-labeled by intraperitoneal injection of 0.5 mCi of L-[<sup>35</sup>S]methionine in 0.5 ml of PBS. 2 h later, a chase consisting of 2.5 ml of 20 mg/ml unlabeled L-methionine in PBS was administered intraperitoneally, and additional chases (2.5 ml each) were injected at 12-h intervals. The adequacy of the initial pulse-labeling period was ascertained in separate experiments where maximal incorporation of radioactivity into plasma membrane bound cBSTP was found after 2 h. Furthermore, varying the chase intervals between 8 and 12 h demonstrated that 12-h periods were sufficient to prevent late incorporation of newly synthesized radioactive cBSTP into LPM subfractions. 12 h after pulse labeling (10 h after the first chase) the animals were either sham operated (controls), or their bile duct was ligated. After various time intervals (zero time = pulse labeling), the rats were killed, their livers were excised, and mixed LPM were isolated in parallel from six individual livers (see above). cBSTP was quantitatively immunoprecipitated from each mixed LPM preparation. The immunoprecipitates were dissolved in 5 ml of scintillation cocktail (Opti-Fluor, Canberra-Packard International SA, Zurich, Switzerland) and the radioactivity was determined in a liquid scintillation counter (Tri-Carb 460 CD, Canberra-Packard International SA).

**Rocket immunoelectrophoresis.** The content of membrane-bound cBSTP was quantitatively determined in a total particulate fraction (sedimented from homogenates at 100,000 *g*<sub>ave</sub> for 30 min) and in isolated LPM subfractions from control and cholestatic livers by rocket immunoelectrophoresis (29). The electrophoresis was carried out in a flat-bed apparatus (FBE 3000, Pharmacia/LKB, Dübendorf, Switzer-

land) using 93 × 84-mm gel-bond slides that were covered with 1% agarose in electrophoresis buffer containing 91.5 mM Tris, 30.4 mM diethylbarbituric acid, 4 mM Ca<sup>2+</sup>-lactate, 2.5 mM NaN<sub>3</sub>, 0.2% Triton X-100, 9.7 mM Na<sup>+</sup>-cholate and 1% (vol/vol) rabbit anti-cBSTP serum. LPM samples were solubilized in electrophoresis buffer without anti-cBSTP serum, and sample volumes between 2.5–7.5 μl (25–100 μg of protein) were applied into holes punched into the agarose slides 1 cm apart from the cathodic side. Electrophoresis was performed at 80 V for 20 h at 15°C. Subsequently the agarose plates were equilibrated with 250 ml PBS for 4 h to remove nonprecipitated proteins. After drying (20°C for 5 h) the plates were stained in 20% methanol, 7.5% acetic acid, and 0.25% Serva Blue S (Serva, Heidelberg, Federal Republic of Germany) for 30 min and destained in 20% methanol, 7.5% acetic acid for at least 2 h. The content of cBSTP in the various samples was estimated from the planimetrically determined area under the rocket-shaped precipitation lines.

**Immunolocalization of cBSTP.** For immunofluorescence studies, liver tissue samples were snap frozen in liquid nitrogen. Cryostat sections (5 μm) were fixed for 10 min in cold acetone (0–4°C) and air dried. After rehydration the sections were sequentially incubated for 20 min at room temperature in each of the following reagents: (a) normal goat serum (1:5 dilution, Miles Scientific, Inc., Naperville, IL), (b) rabbit anti-cBSTP serum (1:100 dilution), and (c) FITC-conjugated goat anti-rabbit serum IgG (1:20 dilution, Miles Scientific, Inc.). All dilutions and rinsing steps between incubations were performed with Tris-buffered saline (pH 7.6). Controls for nonspecific fluorescence were prepared by replacing anti-cBSTP serum with preimmune serum at the same concentration. Slides were coverslipped with glycerol and examined immediately.

For immunogold labeling, livers were perfusion fixed with freshly prepared paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Small tissue blocks (0.5–1 mm) were cryoprotected with 2.3 M sucrose and rapidly frozen in liquid propane. Frozen semithin sections (1 μm) were cut on a Reichert FC 4E Ultracut, mounted on chromalum gelatine coated slides, and incubated at room temperature sequentially in (a) normal goat serum (1:50 dilution, 10 min), (b) rabbit anti-cBSTP serum (1:500 dilution, 1 h), and (c) goat anti-rabbit IgG-coated colloidal gold (5 nm, 1:50 dilution, 1 h, Janssen, Olen, Belgium). All dilutions and rinsing steps were performed with PBS. The gold label was visualized with a silver enhancement kit (IntenSE M, Janssen). Some sections were counterstained with Mayer's hematoxylin. After dehydration slides were coverslipped with Entellan (Merck & Co., Darmstadt, Federal Republic of Germany). Controls for nonspecific label were prepared by replacing anti-cBSTP serum with preimmune serum or with PBS.

Micrographs were taken with standardized shutter speeds on a Photomicroscope II (Carl Zeiss-Schweiz Inc., Zurich, Switzerland) equipped with epifluorescence illumination using 450–490-nm exciter and 515–565-nm barrier filters in combination with a 510-nm dichromatic beam splitter or on a bright-field microscope (Axiophot, Carl Zeiss, Inc.). Paired prints were exposed for identical time periods.

**Vesicle transport studies.** Frozen membrane suspensions were quickly thawed by immersion of the tubes in a 37°C waterbath, diluted to the desired protein concentration (2.5–10 mg/ml), revesiculated by 20 passages through a 25-gauge needle and kept on ice until used. Preloading of cLPM vesicles was performed by adding [<sup>3</sup>H]-taurocholate (10 μM) before revesiculation followed by preincubation of the vesicles at 25°C for 20 min. Where indicated the vesicles were also treated with valinomycin (10 μg/mg protein; Figs. 4 and 5). [<sup>3</sup>H]-taurocholate efflux from cLPM vesicles and tracer uptake into bLPM vesicles were determined by a rapid filtration technique as previously described (12, 13). The exact incubation conditions are given in the corresponding figure legends (Figs. 4–6). After the indicated time intervals the reactions were stopped by addition of 2.5 ml of ice-cold stop solution consisting of 100 mM K<sup>+</sup>, 100 mM sucrose, 5 mM Mg<sup>2+</sup>, 0.2 mM Ca<sup>2+</sup>, 110.4 mM gluconate, 10 mM Hepes/Tris, pH 7.5. Vesicle-associated ligand was separated from free ligand by rapid filtration through a 0.45-μm nitrocellulose filter (Sartorius, Göttingen, Federal

Republic of Germany) that was preincubated in and additionally pre-washed with 1 mM taurocholate (2 ml) to reduce nonspecific filter binding. After two washes with 2.5 ml of cold stop solution, the filters were dissolved in 5 ml of scintillation fluid (Filter Count, Canberra-Packard International SA). All measurements were performed in quadruplicates in at least two different membrane preparations.

## Results

**Characteristics of cholestatic animals.** After 50 h, body weights were similar in sham-operated (231 ± 11 g; means ± SD, *n* = 12) and bile duct-ligated (226 ± 12 g) animals whereas liver weights of bile duct-ligated rats were slightly decreased from 14.3 ± 1.6 to 11.6 ± 1.1 g (*P* < 0.01), respectively. In serum, bile duct ligation increased the concentrations of bile acids to 48.3 ± 4.2 μmol/liter (controls: 2.0 ± 0.5 μmol/liter), total bilirubin to 99 ± 29 μmol/liter (controls: 2.5 ± 0.8 μmol/liter), and alkaline phosphatase to 525 ± 131 U/liter (controls: 354 ± 70 U/liter). Light and transmission electron microscopy showed qualitatively the well-known alterations (5). The most prominent changes occurred at the secretory pole of hepatocytes including dilatation of bile canaliculi, distortion and reduction of canalicular microvilli, and thickening of the pericanalicular ectoplasma containing numerous vesicles (see Fig. 3 D).

**Enzymatic characterization of LPM subfractions isolated from normal and cholestatic rat liver.** For correct interpretation of the vesicle studies outlined below, it was necessary to verify that comparable bLPM and cLPM subfractions could be purified from sham-operated and bile duct-ligated animals. Under both conditions total protein recoveries from 10 g of liver were identical for bLPM (controls, 3.4 ± 0.4 mg, 50 h; bile duct ligation, 3.4 ± 0.5 mg; means ± SD, *n* = 12) whereas those for cLPM were slightly reduced from 2.6 ± 0.3 mg protein in controls to 1.9 ± 0.4 mg in bile duct-ligated (50 h) animals. Na<sup>+</sup>K<sup>+</sup>ATPase activity was slightly, but insignificantly increased in homogenates after 50 h of bile duct ligation (Table I). However, significant 20–30% increases of Na<sup>+</sup>K<sup>+</sup>ATPase activity were found in homogenates and mixed LPM at 14 and 26 h after surgery (data not shown). Based on Na<sup>+</sup>K<sup>+</sup>ATPase activity (17, 30–33) and on marker enzyme activities for mitochondria (succinate cytochrome *c* reductase) lysosomes (acid phosphatase) and Golgi membranes (galactosyltransferase), bile duct ligation for 50 h did not prevent successful purification and separation of bLPM and cLPM vesicles by sucrose-density centrifugations (Table I). In addition, contaminations with endoplasmic reticulum membranes (NADPH cytochrome *c* reductase) were low, although “cholestatic” bLPM were approximately twofold more contaminated with microsomes (relative enrichment [RE] 0.8) compared with “normal” bLPM (RE 0.4; Table I). The most significant differences in enzyme activities were obtained for the canalicular markers Mg<sup>2+</sup>ATPase, leucineaminopeptidase, alkaline phosphatase, and γ-glutamyltranspeptidase (Table I). Alkaline phosphatase activity was 2.2-fold higher in homogenates of cholestatic compared with control livers, which most probably reflects enzyme induction (34, 35). Although alkaline phosphatase activity was 1.7-fold less enriched in cholestatic cLPM vesicles (RE 42 vs. 70 in controls), it still exhibited a 33% higher absolute specific activity (16.8 μmol · mg<sup>-1</sup> · h<sup>-1</sup>) compared with normal cLPM (12.6 μmol · mg<sup>-1</sup> · h<sup>-1</sup>). In contrast, Mg<sup>2+</sup>ATPase, leucineaminopeptidase, and γ-glutamyltranspeptidase activities were significantly decreased in cLPM of

Table I. Enzyme Enrichment and Percent Recovery in LPM Subfractions from Normal and Cholestatic Rat Liver\*

	Homogenates (specific activity) <sup>‡</sup>		(RE) <sup>§</sup>					
			Mixed LPM		bLPM		cLPM	
	Control	Cholestasis	Control	Cholestasis	Control	Cholestasis	Control	Cholestasis
Na <sup>+</sup> /K <sup>+</sup> -ATPase	0.66±0.20	0.82±0.38	16±7	12±5	30±9	23±11	2±0.1	2±0.2
Mg <sup>2+</sup> -ATPase	2.20±0.70	2.42±0.44	6±2	6±2	6±2	4±0.3	46±10	9±0.5 <sup>  </sup>
Leucineaminopeptidase	3.50±0.90	3.21±1.15	4±2	1±0.5	2±1	2±0.1	23±4	13±0.7 <sup>  </sup>
Alkaline phosphatase	0.18±0.02	0.40±0.21	13±2	18±5	8±3	5±1	70±15	42±4 <sup>  </sup>
γ-Glutamyltranspeptidase	0.03±0.01	0.02±0.01	6±2	5±2	5±1	5±2	27±2	15±2 <sup>  </sup>
Succinate-cytochrome c-reductase	4.60±2.10	3.10±0.60	2.5±0.7	3.6±0.5 <sup>  </sup>	1.5±0.3	1.7±0.6	0.2±0.1	0.2±0.03
NADPH-cytochrome c-reductase	1.84±0.70	1.50±0.60	0.4±0.1	0.6±0.2 <sup>  </sup>	0.4±0.1	0.8±0.1	1.3±0.2	1.1±0.2
Acid phosphatase	0.41±0.10	0.40±0.12	0.8±0.3	0.9±0.4	0.4±0.1	0.4±0.2	0.8±0.2	0.7±0.2
Galactosyltransferase	13.2±5.80	15.0±4.0	0.2±0.1	0.3±0.2	ND	ND	ND	ND

ND, not detectable. \* LPM subfractions were isolated 50 h after sham operation (controls) or bile duct ligation (cholestasis), respectively.

<sup>‡</sup> All data represent the means±SD of single measurements in six separate membrane preparations (*n* = 6). Specific activities are expressed as μmol product formed · mg<sup>-1</sup> protein · h<sup>-1</sup>, except for galactosyltransferase for which the corresponding units are nmol galactose transferred · mg<sup>-1</sup> · h<sup>-1</sup>. <sup>§</sup> Relative enrichment (RE) is defined as the ratio of specific activity in the LPM to specific activity in the homogenate.

<sup>||</sup> Significantly different from controls (*P* < 0.05) as determined by paired Student *t* test.

cholestatic liver by 88%, 49%, and 63%, respectively (Table I). Furthermore, as shown in Table II recoveries of canalicular marker enzyme activities were uniformly decreased in cholestatic cLPM, suggesting loss of canalicular membranes either in vitro during subcellular fractionation or in vivo because of bile duct ligation (see below). In bLPM, enrichment and recovery of canalicular marker enzyme activities were either similar (leucineaminopeptidase, γ-glutamyltranspeptidase) or only slightly decreased (Mg<sup>2+</sup>ATPase, alkaline phosphatase) in cholestatic compared with control livers (Tables I and II). These data might reflect (but do not prove) some accumulation of canalicular marker enzymes at the basolateral plasma membrane during obstructive cholestasis (8–10) rather than increased contamination of cholestatic bLPM with cLPM, since the protein recovery was similar in both bLPM subfractions (see above). Thus, it is concluded from the data in Tables I and II that with the exception of a slightly higher microsomal contamination of cholestatic bLPM, the degree of purification

Table II. Enzyme Recovery in bLPM and cLPM Subfractions from Normal and Cholestatic Rat Liver\*

	Recovery <sup>‡</sup>			
	bLPM		cLPM	
	Control	Cholestasis	Control	Cholestasis
	%	%	%	%
Na <sup>+</sup> K <sup>+</sup> -ATPase	8.0±0.8	6.3±0.8 <sup>§</sup>	0.4±0.2	0.3±0.3
Mg <sup>2+</sup> -ATPase	1.6±0.2	1.1±0.2 <sup>§</sup>	9.4±1.2	1.4±0.3 <sup>§</sup>
Leucineaminopeptidase	0.5±0.2	0.6±0.2	4.7±0.6	2.0±0.4 <sup>§</sup>
Alkaline phosphatase	2.1±0.3	1.4±0.5 <sup>§</sup>	14.6±1.6	6.4±1.3 <sup>§</sup>
γ-Glutamyltranspeptidase	1.3±0.3	1.4±0.4	5.5±0.5	2.3±0.4 <sup>§</sup>

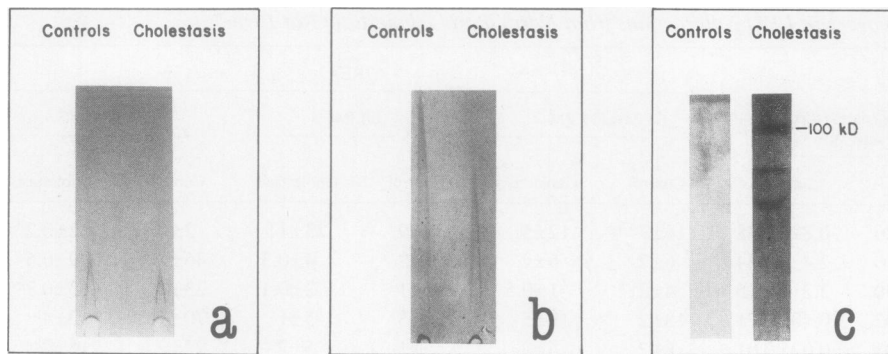
\* LPM subfractions were isolated 50 h after sham operation (controls) or bile duct ligation (cholestasis), respectively. All data represent the means±SD of single measurements in six separate membrane preparations (*n* = 6).

<sup>‡</sup> Total activities in homogenates correspond to 100%.

<sup>§</sup> Significantly different from controls (*P* < 0.05) as determined by paired Student *t* test.

was otherwise similar for bLPM and cLPM subfractions of normal and cholestatic rat livers.

*Effect of bile duct ligation on turnover and subcellular distribution of membrane-bound cBSTP.* To characterize more specifically the canalicular membrane alterations during extrahepatic obstructive cholestasis we first determined the in vivo degradation half-life of cBSTP by pulse-chase experiments with [<sup>35</sup>S]methionine. As demonstrated in Fig. 1, bile duct ligation decreased the degradation half-life of cBSTP from 65±5 h (means±SD, *n* = 3) in controls to 25±3 h in cholestatic animals. In these experiments in vivo-labeled cBSTP was immunoprecipitated from mixed LPM, and the radioactivity in immunoprecipitates was determined by liquid scintillation counting. However, identical results were also obtained if the immunoprecipitates were first subjected to SDS-PAGE and the radioactivity of the 100-kD band then determined by fluorography (data not shown). Thus, the results suggest an increased overall degradation of cBSTP in bile duct-ligated animals. This conclusion was further supported by the presence of decreased amounts of membrane bound cBSTP in the total particulate fraction as well as in mixed LPM and cLPM subfractions of cholestatic livers as determined by rocket immunoelectrophoresis (Fig. 2 and Table III). Thereby, the accelerated breakdown was accompanied by some release of cBSTP into cholestatic bile either in its intact form (mol wt 100,000) or as partially degraded (e.g., deglycosylated) products with apparent molecular weights of ~ 60,000 and 50,000 (Fig. 2). Most interestingly, bile duct ligation exerted different effects on cBSTP contents in bLPM and cLPM subfractions. Thus, while in the latter cBSTP was decreased by ~ 80%, there was a surprising increase of immunoreactive cBSTP by ~ 55% in cholestatic bLPM (Table III). Since the extent of this increase cannot be explained by a higher contamination of cholestatic bLPM with canalicular membranes (Tables I and II), the data suggest a cholestasis-induced accumulation of cBSTP at the basolateral plasma membrane. This possibility was further investigated in the intact liver by indirect immunofluorescence techniques.



**Figure 2.** Content of membrane-bound cBSTP in (a) homogenate, (b) cLPM, and (c) bile of normal and cholestatic rat liver. Rats were killed 50 h after sham operation (controls) or bile duct ligation (cholestasis). The total particulate fraction was sedimented from homogenates at 100,000  $g_{ave}$  for 30 min. cLPM were isolated as described in Methods. Aliquots for total particulate fraction (200  $\mu$ g of protein) and cLPM vesicles (50  $\mu$ g) were subjected to rocket immunoelectrophoresis. Bile was collected from the common bile duct and total biliary pro-

teins were precipitated from 50- $\mu$ l bile samples with 20  $\mu$ l of 3 M trichloroacetic acid. The bile proteins were separated by SDS-PAGE and cBSTP-related proteins were detected by immunoblotting.

*In vivo distribution of cBSTP in normal and cholestatic rat liver.* As demonstrated in Fig. 3, immunoreactive cBSTP was almost exclusively concentrated in bile canaliculi of normal liver. Only weak immunoreactivity was detected at the sinusoidal border of normal hepatocytes. In contrast, 50 h after bile duct ligation immunoreactivity was reduced at the bile canalicular and significantly increased at the basolateral plasma membrane domains. Identical results were obtained with a more sensitive silver enhanced immunogold labeling technique (Fig. 3, C and D). In addition, this method demonstrated the presence of numerous immunopositive intracellular spots near the bile canaliculi probably representing cBSTP containing vesicles. These in situ findings corroborate the in vitro studies described above and indicate that portions of cBSTP molecules accumulate in intracellular vesicles and at the basolateral membrane during extrahepatic obstructive cholestasis. To delineate the functional significance of increased basolateral cBSTP content, we next performed taurocholate transport studies in isolated normal and cholestatic cLPM and bLPM vesicles.

*Taurocholate transport properties of normal and cholestatic cLPM and bLPM vesicles.* It has been previously shown that carrier-mediated taurocholate anion uptake into (36) as well as efflux from (12, 13) cLPM vesicles is independent of  $Na^+$  and

can be stimulated by intravesicular positive or negative  $K^+$  diffusion potentials, respectively. Therefore, we first compared the effects of an inside negative  $K^+$  diffusion potential on taurocholate efflux from normal and cholestatic cLPM vesicles. As shown in Fig. 4, cLPM vesicles from bile duct-ligated (50 h) animals exhibited markedly reduced potential-sensitive taurocholate efflux as compared with cLPM vesicles from sham-operated controls. In contrast, an intravesicular positive  $K^+$  diffusion potential markedly stimulated  $Na^+$ -independent taurocholate uptake into cholestatic bLPM vesicles, whereas it exerted only minimal effects on early taurocholate uptake rates in bLPM vesicles of normal liver (Fig. 5). This increased electrogenic taurocholate uptake into cholestatic bLPM could quantitatively not be accounted for by increased microsomal contamination (Table I), as verified in separate experiments with microsomes isolated from normal and cholestatic livers (data not shown). Thus, the above demonstrated basolateral accumulation of immunoreactive cBSTP during obstructive cholestasis (Table III and Fig. 3) was functionally paralleled by increased canalicular taurocholate transport properties in bLPM vesicles indicating basolateral accumulation of functionally intact cBSTP in cholestatic hepatocytes. For control, we finally also determined  $Na^+$ -gradient driven, secondary active taurocholate uptake, a typical basolateral function of normal hepatocytes (ref. 12, 37–39), in bLPM vesicles of normal and cholestatic (50-h bile duct ligation) liver. In these experiments taurocholate uptake was determined in the presence of out to in  $NaCl$  or  $KCl$  gradients (Fig. 6). Thereby, the formation of an inside positive  $K^+$  diffusion potential was prevented by not treating the vesicles with valinomycin and by using the permeant anion chloride rather than gluconate. The data in Fig. 6 demonstrate that an inwardly directed  $NaCl$ , but not  $KCl$ , gradient induced a similar transient intravesicular accumulation of [ $^3H$ ]taurocholate (overshoot) in both bLPM preparations. Together with the unaltered  $Na^+K^+$ ATPase activity (Table I), these results suggest that bile duct ligation for up to 50 h does not affect sodium-dependent uptake of bile salts into rat hepatocytes.

**Table III.** Contents of cBSTP in Subcellular Fractions Isolated from Normal and Cholestatic Rat Liver\*

	Amount of cBSTP	
	Controls	Cholestasis
	pmol/mg protein	
Total particulate fraction <sup>‡</sup>	127 $\pm$ 7	77 $\pm$ 5 <sup>§</sup> (-39%)
Mixed LPM	498 $\pm$ 9	154 $\pm$ 5 <sup>§</sup> (-69%)
bLPM	147 $\pm$ 9	228 $\pm$ 47 <sup>§</sup> (+55%)
cLPM	855 $\pm$ 135	191 $\pm$ 73 <sup>§</sup> (-78%)

\* Subcellular fractions were isolated 50 h after sham operation (controls) or bile duct ligation (cholestasis), respectively. cBSTP was quantitated by rocket immunoelectrophoresis. All data represent the means $\pm$ SD of single determinations in three separate membrane preparations.

<sup>‡</sup> The "total particulate fraction" corresponds to the material sedimented from homogenates at 100,000  $g_{ave}$  for 30 min.

<sup>§</sup> Significantly different from controls as determined by paired Student *t* test ( $^8P < 0.001$ ).

## Discussion

In the present study we studied the effects of extrahepatic mechanical obstruction of the common bile duct on the bile salt secretory polarity of rat hepatocytes. Specifically, the fate and functional activity of the cBSTP were investigated in intact liver as well as isolated basolateral (bLPM) and canalicular

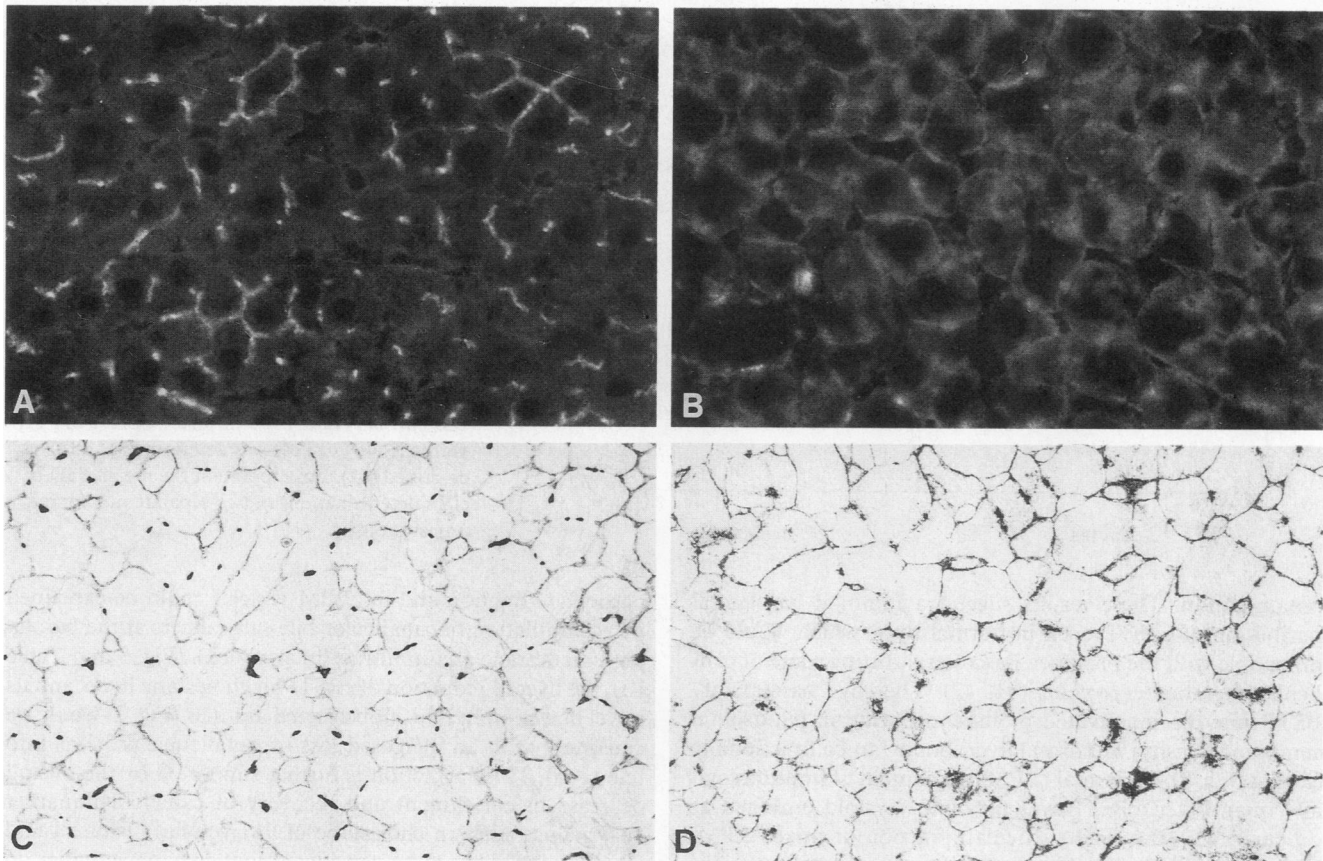


Figure 3. In situ immunolocalization of cBSTP in (A and C) normal and (B and D) cholestatic rat liver. (A and B) Immunofluorescence; (C and D) silver enhanced immunogold labeling. Cholestasis was induced by bile duct ligation for 50 h.  $\times 400$ .

(cLPM) membrane vesicles. The findings demonstrate both an increased degradation and loss of cBSTP into bile (Figs. 1 and 2; Table III) as well as an accumulation of functionally active cBSTP at the basolateral membrane domain of hepatocytes (Figs. 3–5; Table III) in bile duct-ligated animals. Although previously suggested on indirect morphological grounds (2, 8–11), the present study represents the first direct demonstration of a reversed bile salt secretory polarity in cholestatic hepatocytes. Furthermore, in view of similar  $\text{Na}^+$ -dependent taurocholate uptake values in bLPM vesicles of normal and

cholestatic livers (Fig. 6), our results support the hypothesis (2) that bile salts and possibly also other bile constituents can regurgitate into blood plasma without being first secreted into bile canaliculi and obstructed bile ducts (Fig. 7).

In normal liver cBSTP is almost exclusively distributed to the canalicular membrane of hepatocytes (Fig. 3). However, faint immunolabel is consistently found on the basolateral membrane domain as well (Fig. 3 and reference 40) indicating that the relatively high specific cBSTP content of “normal” bLPM (Table III) did not solely result from canalicular cross-

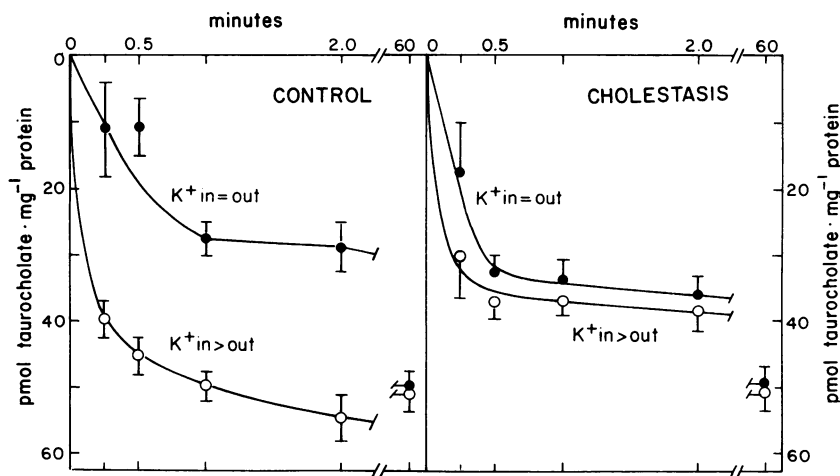
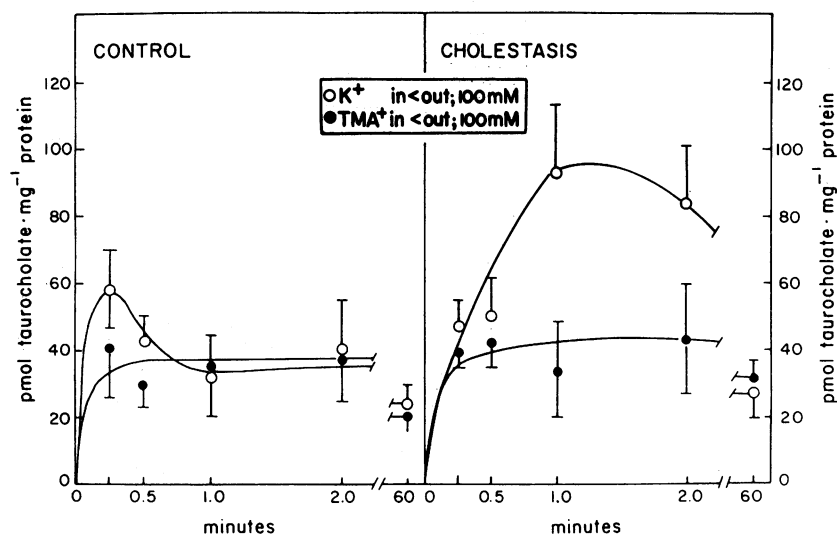


Figure 4. Potential-sensitive efflux of taurocholate from cLPM vesicles of normal and cholestatic rat liver. Animals were killed 50 h after sham operation (controls) or bile duct ligation (cholestasis). Isolated cLPM vesicles were resuspended in a medium containing 100 mM  $\text{K}^+$ , 100 mM sucrose, 0.2 mM  $\text{Ca}^{2+}$ , 5 mM  $\text{Mg}^{2+}$ , 110.4 mM gluconate, and 20 mM HEPES/Tris, adjusted to pH 7.5. The vesicles were preloaded with 10  $\mu\text{M}$  [ $^3\text{H}$ ]-taurocholate, treated with valinomycin (10  $\mu\text{g}/\text{mg}$  protein) and 10- $\mu\text{l}$  aliquots (100  $\mu\text{g}$  of protein) incubated in 190  $\mu\text{l}$  of incubation medium containing 100 mM sucrose, 0.2 mM  $\text{Ca}^{2+}$ , 5 mM  $\text{Mg}^{2+}$ , 110.4 mM gluconate, 20 mM HEPES/Tris, pH 7.5, and either (●) or (○) 100 mM tetramethylammonium. Tracer taurocholate efflux was determined at 25°C as described (12, 13). Data represent the means  $\pm$  SD of eight determinations in two separate membrane preparations.



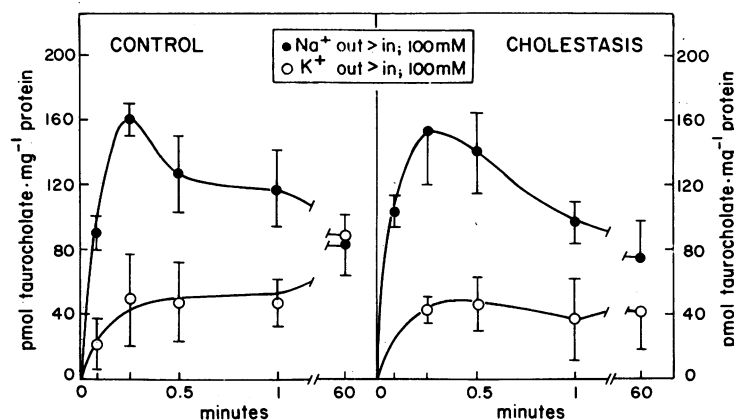


**Figure 5.** Electronic Na<sup>+</sup>-independent taurocholate uptake in bLPM vesicles of normal and cholestatic rat liver. Rats were killed 50 h after sham-operation (controls) or bile duct ligation (cholestasis). Isolated bLPM vesicles were resuspended in a medium containing 250 mM sucrose, 0.2 mM Ca<sup>2+</sup>, 5 mM Mg<sup>2+</sup>, 10.4 mM gluconate, and 10 mM HEPES/Tris, adjusted to pH 7.5. The vesicles were treated with valinomycin (10 μg/mg protein) and 20-μl aliquots (50 μg of protein) incubated in 80 μl of incubation medium containing 50 mM sucrose, 0.2 mM Ca<sup>2+</sup>, 5 mM Mg<sup>2+</sup>, 110.4 mM Cl<sup>-</sup>, 10 mM HEPES/Tris, pH 7.5, and either (●) 100 mM Na<sup>+</sup> or (○) 100 mM K<sup>+</sup>. Uptake of [<sup>3</sup>H]-taurocholate (10 μM) was determined at 25°C as described (12). Data represent the means±SD of eight determinations in two separate membrane preparations.

contamination. These results suggest a minimal basolateral distribution of cBSTP even in normal liver, which would be compatible with the proposed basolateral sorting route of canalicular membrane proteins (41, 42). Thereby "basolateral" cBSTP may be inactivated or its concentration (sinusoidal membrane density) in normal liver too low to be functionally relevant (Fig. 5). In normal cLPM, the content of immunologically quantitated cBSTP was 6.8- and 1.7-fold enriched as compared with the total particulate fraction or mixed LPM, respectively (Table III). Thus, even if one corrects for the absence of soluble proteins in the total particulate fraction (50% of homogenate), the cLPM enrichment factors for cBSTP were considerably lower than those for the canalicular marker enzyme activities (Table I). The reasons for these discrepancies are unknown at present, but a possibility may be that cBSTP exhibits significant additional subcellular localization such as for example in endoplasmic reticulum (43, 44) and/or in Golgi complex (45).

Bile duct ligation resulted in a 60% decrease of the *in vivo* half-life of cBSTP immunoprecipitated from mixed LPM (Fig. 1) and in an overall reduction of membrane bound cBSTP by 39% in homogenates (total particulate fraction) and by 78% in cLPM vesicles (Fig. 2 and Table III). Simultaneously, the electrogenic taurocholate efflux was reduced by > 70% in cholestatic cLPM vesicles compared with controls (Fig. 4). Although the discrepancy between the reduction of cBSTP in the total

particulate fraction and in cLPM vesicles could be explained by accumulation of canalicular bile salt carriers at the basolateral membrane and in intracellular vesicles (Fig. 3 and Table III), the overall reduction of cBSTP in cholestatic livers and its novel detection in bile of obstructed animals (Fig. 2) would be consistent with an increased loss of canalicular proteins into bile (1-5). This suggestion is further supported by the overall decrease in enrichment and recovery of canalicular marker enzyme activities in cholestatic cLPM vesicles (Tables I and II), although it has to be considered that enzyme activities do not necessarily reflect amounts of enzyme protein. Nevertheless, the findings are in agreement with the reported preferential canalicular loss of intramembrane particles in freeze fracture replicas during bile duct obstruction (46). Interestingly, the ~ 80% reduction in content and functional activity of cBSTP in cholestatic cLPM vesicles (Table III and Fig. 4) was associated with a minor decrease in the recovery of cLPM protein (-30%) and with a 50-60% reduction in the activity and recovery of the canalicular membrane enzymes leucineaminopeptidase and γ-glutamyltranspeptidase (Tables I and II). Because of the low overall cLPM recovery it remains unclear whether these differences resulted from selection of a special subpopulation of cLPM vesicles during subcellular fractionation, or whether they rather reflect a preferential loss *in vivo* of cBSTP-enriched canalicular membrane domains. Although the latter possibility would be compatible with the reported



**Figure 6.** Na<sup>+</sup> gradient-dependent taurocholate uptake into bLPM vesicles of normal and cholestatic rat liver. Rats were killed 50 h after sham operation (controls) or bile duct ligation (cholestasis). Isolated bLPM vesicles were resuspended in a medium containing 250 mM sucrose, 0.2 mM Ca<sup>2+</sup>, 5 mM Mg<sup>2+</sup>, 10.4 mM gluconate, and 10 mM HEPES/Tris, adjusted to pH 7.5. Aliquots of 20 μl (50 μg of protein) were incubated in 80 μl of incubation medium containing 50 mM sucrose, 0.2 mM Ca<sup>2+</sup>, 5 mM Mg<sup>2+</sup>, 110.4 mM Cl<sup>-</sup>, 10 mM HEPES/Tris, pH 7.5, and either (●) 100 mM Na<sup>+</sup> or (○) 100 mM K<sup>+</sup>. Uptake of [<sup>3</sup>H]-taurocholate (10 μM) was determined at 25°C as described (12). Data represent the means±SD of eight determinations in two separate membrane preparations.

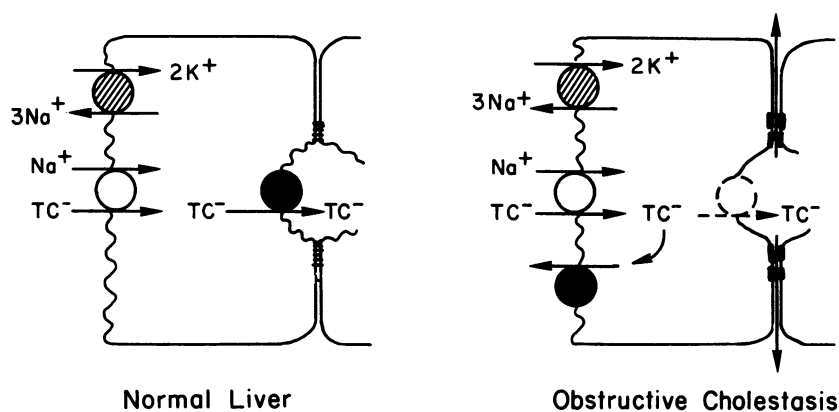


Figure 7. Schematic illustration of the effect of obstructive cholestasis on the hepatocellular surface distribution of cBSTP. For explanations see text. TC<sup>-</sup>, taurocholate.

reduction of bile salt secretion shortly after relief of prolonged bile duct obstruction (47), a decreased amount of cBSTP within the canalicular membrane could obviously not explain the observation of a normal taurocholate transport maximum after relief of a 7-d bile duct-ligated period (48). However, decreased hepatocellular secretion of taurocholate during biliary obstruction might be a transient phenomenon as recently evidenced by a histoautoradiographic study in rat liver (49). Clearly further studies are warranted to investigate whether reestablishment of hepatocellular bile acid secretion during and after prolonged biliary obstruction quantitatively correlates with a concomitant canalicular increase of functionally active cBSTP.

In addition to increased degradation, bile duct ligation also resulted in an accumulation of cBSTP within the basolateral border of hepatocytes as evidenced in both intact liver (Fig. 3) as well as isolated bLPM vesicles (Table III). In the latter, the 55% increase in membrane-associated cBSTP was paralleled by a similar increase (between 50% and 100%) in potential sensitive taurocholate anion uptake (Fig. 5). These findings indicate basolateral accumulation of functionally active canalicular bile salt carriers in cholestatic hepatocytes rather than an increased contamination of "cholestatic" bLPM vesicles with canalicular membranes, since the relative enrichments of canalicular marker enzyme activities remained similar in bLPM vesicles of normal and cholestatic liver (Table I). Thus, the results provide direct evidence for structural and functional reversal of the bile salt secretory polarity in hepatocytes during extrahepatic cholestasis, thereby corroborating previous enzymatic, histochemical and immunofluorescence studies with various canalicular enzymes (2, 8–10) and with different domain specific antigens of unknown functions (11), respectively. These earlier observations indicate that loss of the hepatocellular surface polarization during biliary obstruction is a general phenomenon that affects a variety of both canalicular (8–11) and sinusoidal (11) domain specific proteins. The present study provides no conclusive evidence with respect to an altered polarization of plasma membrane constituents other than cBSTP in cholestatic hepatocytes, although in cholestatic bLPM canalicular marker enzyme activities were considerably less decreased compared with cholestatic cLPM or even unaltered when compared with normal bLPM (Tables I and II). Thus, differential cholestatic effects on various enzymes (Tables I and references 9, 34, 35, and 50) and/or the low overall recovery of isolated basolateral and canalicular membranes (Table II) might have masked the clear detection of a simultaneous basolateral accumulation of the respective

marker enzyme activities. Yet, the unaltered Na<sup>+</sup>K<sup>+</sup>ATPase activity (Table I and reference 9) and the normal Na<sup>+</sup>-dependent taurocholate uptake (Fig. 6) in cholestatic bLPM vesicles strongly indicate that essential basolateral membrane functions remain intact during the initial period of obstructive cholestasis. Hence, as schematically outlined in Fig. 7, the basolateral membrane could exhibit normal Na<sup>+</sup>-dependent bile salt uptake as well as cBSTP-mediated potential sensitive bile salt anion efflux in cholestatic hepatocytes. The latter process might protect hepatocytes from too high an accumulation of toxic bile salts within the cell interior and could temporarily well sustain high bile salt concentrations in blood plasma even after relief of the biliary obstruction (48).

Several mechanisms could potentially explain the basolateral accumulation of cBSTP in hepatocytes of bile duct-ligated animals. First, cBSTP could have diffused in the plane of the membrane across altered tight junctions (51–53), although it should be realized that passage of proteins across tight junctions has never been conclusively demonstrated. Secondly, cBSTP could have reached the basolateral membrane transcellularly via retrograde diacytosis (6, 7). Thirdly, newly synthesized cBSTP could have been missorted in the Golgi apparatus (54) followed by incorrect insertion into the plasma membrane. Fourthly, based on the recent suggestion that canalicular membrane biogenesis first requires insertion of integral canalicular membrane proteins into the basolateral plasma membrane followed by their selective endocytosis and transport to the canalicular domain (41, 42) obstructive cholestasis could have selectively blocked the basolateral-to-canalicular movement of cBSTP by affecting some form of vesicle-mediated transcytosis. Whatever the exact mechanism, the evidence for the presence of cBSTP containing intracellular vesicles suggests that a vesicle-mediated transport pathway is disrupted during obstructive cholestasis. Clearly further experimentation is required to delineate the exact mechanisms involved in the establishment of the reversed bile salt secretory polarity in cholestatic hepatocytes.

*Note added in proof.* Recent studies performed to further characterize cBSTP provided evidence that our polyclonal antiserum recognizes at least three different 100-kD canalicular proteins. Since it is unclear at present which of these protein(s) is (are) specifically involved in canalicular secretion of bile acids, the reported immunological studies might quantitatively be overestimated. However, these limitations should not alter the main conclusions of the present work regarding the reversal of the bile salt secretory polarity of hepatocytes during extrahepatic obstructive cholestasis.



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