Physicochemical and Immunohistological Studies of a Sulfobromophthalein- and Bilirubin-binding Protein from Rat Liver Plasma Membranes

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ABSTRACT Affinity chromatography over bilirubin-agarose and sulfobromophthalein (BSP)-agarose was used to isolate two proteins, with high affinities for bilirubin and BSP, respectively, from Triton X-100-solubilized rat liver plasma membranes. The protein eluted from either affinity column migrated as a single band of ~55,000 D on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and either protein cochromatographed with both [^{14}C]bilirubin and [^{35}S]BSP on Sephadex G-75. On gradient gels without reduction or SDS, or on Sephadex G-150, the native BSP-binding protein had an estimated molecular mass of ~100,000 D. After incubation with SDS, an additional Sephadex G-150 peak of molecular mass of 56,000 D was observed. Both, the 100,000- and 56,000-D G-150 peaks cochromatographed with [^{35}S]BSP. The native protein had an isoelectric point of 3.5, stained with periodic acid-Schiff but not Sudan black, and contained 4 mol of sialic acid per mol of protein. A rabbit antibody to the BSP-binding protein gave a line of identity with both the BSP- and bilirubin-binding antigens, and inhibited the binding of [^{14}C]bilirubin and [^{35}S]BSP, but not [^{14}C]oleate or [^{14}C]taurocholate, to rat liver plasma membranes. Immunohistochemical studies revealed the presence of the antigen on all surface domains of rat hepatocytes, but not on other cell populations from normal rat liver. It was not found in other organs. These data are compatible with the hypothesis that a specific liver cell plasma membrane protein mediates the hepatocytic sequestration of bilirubin and BSP.

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INTRODUCTION

The mechanisms by which low-molecular mass cholephilic anions, such as bilirubin and sulfobromophthalein (BSP)¹, cross the liver cell plasma membrane to enter the hepatocyte remain unclear. The kinetics of their hepatocellular uptake in vivo (1, 2) in the isolated, perfused rat liver (3) and in isolated rat hepatocytes (4, 5) suggest the existence of a plasma membrane-associated carrier-mediated transport system that is shared by these two compounds. Despite some evidence of overlapping specificities (5-7), this system is distinct from the principal bile acid transport mechanism (1, 8, 9). Studies of rat liver plasma membranes (LPM) have demonstrated the specific binding of BSP to a high-affinity binding site (10), and isolation of liver cell membrane proteins with high affinities for BSP and bilirubin has been reported by several groups (11-14). The present report describes further studies of a BSP-binding membrane protein from rat liver, and the use of a rabbit antibody to this protein to demonstrate both its identity to the bilirubin-binding protein described earlier (13) and its role in the specific binding of BSP and bilirubin to rat LPM. The distribution of this receptor protein was also studied by immunohistochemical techniques in isolated rat hepatocytes and in tissue sections from rat liver and various other organs.

METHODS

Labeled and unlabeled organic anions. Unconjugated [^{14}C]bilirubin (15.9 mCi/mmol), commercially prepared by

¹ *Abbreviations used in this paper:* BSP, sulfobromophthalein; LPM, liver plasma membrane; OABP, organic anion-binding protein; PAS, periodic acid-Schiff.

biosynthesis from δ -[4- 14 C]aminolevulinic acid (15) was purchased from Amersham Corp. (Arlington Heights, IL) and repurified by alumina column chromatography, recrystallization from chloroform/methanol (15), and thin-layer chromatography (16). The final product had a molar extinction coefficient in chloroform of 61,000 at 450 nm, yielded exclusively the α_0 -para-iodoaniline azopigment (17), and migrated as a single band, which contained >96% of the applied radioactivity on thin-layer chromatography (18, 19). Isomer analysis by thin-layer chromatography (16) and fluorography (20) indicated that ~97% of the purified pigment was bilirubin-IX α , with only traces of -III α and -XIII α isomers. [35 S]BSP (100 mCi/mmol; Amersham Corp.), [14 C]taurocholate (52 mCi/mmol, New England Nuclear, Boston, MA), and [14 C]oleate (900 mCi/mmol, New England Nuclear) were all >99% radiochemically pure by thin-layer chromatography, and were used without further purification.

Nonradioactive bilirubin (molar extinction coefficient 59,000–61,000; isomer composition > 92% bilirubin-IX α [16]), oleate, and sodium taurocholate (all from Sigma Chemical Co., St. Louis, MO) and BSP (Sigma Chemical Co. and Hynson, Wescott, and Dunning, Baltimore, MD) were used without further purification.

Preparation of LPM. LPM fractions enriched in sinusoidal components were prepared in batches by differential centrifugation from the livers of 10 160–170-g male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) as previously described (10). In this procedure, membranes harvested at the 1.22–1.18 g/ml interface of the discontinuous sucrose gradient initially described by Song et al. (21) were further purified according to Fisher et al. (22). Three to four LPM batches were pooled, and after protein determination (23), samples containing 10 mg membrane protein were stored at –80°C until needed. Purity of the membrane preparations and contamination with other cell organelles were analyzed both by electron microscopy (24) and determination of marker enzymes for various subcellular fractions (10). For electron microscopic assessment, samples were prepared and examined as previously described (10). All interpretations were those of one of the authors (Dr. M. A. Gerber), an experienced electron microscopist. For enzymatic analysis, 5'-nucleotidase was assayed as a general marker for plasma membranes (25); glucagon-stimulated adenylate cyclase as a constituent of the sinusoidal region of the LPM (26); and glucose-6-phosphatase (27) and succinate dehydrogenase (28) as markers for microsomal and mitochondrial contamination, respectively.

Membrane binding studies. LPM aliquots equivalent to 5 mg membrane protein were incubated in 1.15 ml 0.15 M NaCl/0.02 M phosphate buffer (PBS), pH 7.6, at room temperature for 30 min with [14 C]bilirubin, [35 S]BSP, [14 C]taurocholate, or [14 C]oleate. Preliminary studies established that, under the conditions used, binding of these test substances to LPM was complete within 15 min. The organic anions had previously been diluted to known specific activity with measured amounts of the corresponding nonradioactive compound, and were added to the incubation mixture as a complex with rat serum albumin (Sigma Chemical Co.) in a 1:4 anion/protein molar ratio. Binding of the various anions to LPM was determined from the distribution of radioactivity between membrane pellets and supernatants after four cycles of centrifugation (15,000 *g* for 15 min), resuspension in PBS, and recentrifugation, as previously described (29, 30). Sufficient quantities of each ligand were incubated to establish its maximal binding to LPM (nanomoles ligand per milligram membrane protein) under the conditions of the system used.

Preparation of affinity columns. BSP was coupled to Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) by the epichlorohydrin reaction (31) using a modification of the procedure of Reichen and Berk (13). After coupling overnight, the gel was incubated at room temperature for 2 h with 1 M ethanolamine, pH 8, to block remaining reactive sites and improve the specificity of the adsorbent. Bilirubin was coupled to Sepharose 4B (Pharmacia Fine Chemicals) via a six-carbon spacer arm (13). Both gels were extensively washed with 1 N NaCl in 0.02 M sodium acetate (pH 4.0) and 0.02 M Tris/HCl (pH 9.0). 60 ml of each adsorbent was then packed into adjustable glass columns of 16-mm i.d. (Amicon Corp, Lexington, MA), and equilibrated for at least 24 h with 1 mM NaHCO₃ (pH 7.6) at a flow rate of 20 ml/h.

Isolation of BSP- and bilirubin-binding membrane proteins. Aliquots of LPM containing 500 mg protein were solubilized by gentle stirring for 1 h at 4°C in 1% (vol/vol) Triton X-100 (1 ml/mg membrane protein). After centrifugation for 1 h at 100,000 *g*, detergent was removed from the supernatant by passage over a 135-ml bed volume of Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) in a 1.3 × 100-cm column (Pharmacia Fine Chemicals) at a flow rate of 20 ml/h (13). The protein extract was charged onto the previously prepared BSP-agarose or bilirubin-agarose columns. These were washed with PBS until no further protein (OD₂₈₀) appeared in the washes. The BSP-agarose column was then eluted with 50 μ M BSP, and the bilirubin-agarose column with 8 M urea. The eluates were dialyzed against 1 mM NaHCO₃ (pH 7.6) and concentrated to 1 mg protein/ml by ultrafiltration (Diaflow Membranes PM-2, Amicon Corp.). All of the above procedures were carried out at 4°C. The bilirubin-agarose column was protected from light by an aluminum foil jacket throughout the procedure.

Membrane proteins, which had not bound to the BSP-agarose column, and which were recovered in the initial PBS washes, were repeatedly concentrated by ultrafiltration and passed over BSP-agarose, to ensure complete removal of any BSP-binding membrane protein. This mixture of miscellaneous membrane proteins, at a concentration of 1 mg/ml, was subsequently used for absorption of the antibody prepared against the BSP-binding protein (see below).

Characterization of the BSP- and bilirubin-binding proteins. The protein composition of the eluates was initially studied after reduction with dithiothreitol by sodium dodecyl sulfate (SDS) gel electrophoresis on an 11% polyacrylamide gel. The protein recovered from the BSP-agarose column was also studied without prior reduction and in the absence of SDS on identical polyacrylamide gels (11%), as well as on polyacrylamide gradient gels (PAA 4/30, Pharmacia Fine Chemicals). It was also subjected to analytical isoelectric focusing in polyacrylamide slab gels (2117-Multiphor, LKB, Rockville, MD). The gels were stained with Coomassie Blue (32) to identify the position of the protein bands. Possible contamination of the BSP- and bilirubin-binding membrane proteins was investigated by double-radial immunodiffusion (33), using rabbit antibodies against rat albumin (Cappel Laboratories, Cochranville, PA) and rat liver ligandin (glutathione S-transferase B). The ligandin antibody, as well as a sample of highly purified ligandin, were gifts of Dr. Allan W. Wolkoff, Department of Medicine and Liver Center, Albert Einstein College of Medicine, New York.

The presence of lipid and carbohydrate components was examined by an agar diffusion technique on glass slides, using the Sudan black and periodic-acid Schiff (PAS) reagents, respectively (34). In addition, the presence of sialic acid in the

protein was detected and quantitated as the silylated derivative by chemical ionization mass spectrometry, using the technique of Roboz, Suzuki, and Bekesi (35).

Cochromatography studies. Approximately 100,000 dpm of [^{14}C]bilirubin was dissolved in 0.1 ml 0.25 N NaOH, and added to 1 ml PBS containing 150 μg of the protein isolated from BSP-agarose. [^{35}S]BSP was added, and the solution applied to a $1.6 \times 25\text{-cm}$ column of Sephadex G-75 (Pharmacia Fine Chemicals). Elution was carried out with 0.1 M NaCl/1 mM NaHCO_3 (pH 7.6) at 15 ml/h. Fractions were assayed for protein content by determination of OD_{280} , and for ^{14}C and ^{35}S by liquid scintillation spectrometry (Mark III, Tracor Analytic Inc., Elk Grove Village, IL). Quench corrections were by a channels ratio method using a ^{133}Ba external standard.

In other studies, 700- μg aliquots of the purified BSP-binding protein were incubated with [^{35}S]BSP either alone or in the presence of 3.3% SDS. The samples, in a total volume of 0.3 ml, were applied to a $1 \times 32\text{-cm}$ column (Amicon Corp.) of Sephadex G-150 (Pharmacia Fine Chemicals), and eluted with 0.1 M NaCl/1 mM NaHCO_3 (pH 7.6) at a flow rate of 5.5 ml/h. Effluents, in 0.43-ml fractions, were monitored for protein and ^{35}S content as described above. The column was calibrated with appropriate molecular mass standards (Pharmacia Fine Chemicals).

Preparation of antibody to BSP-binding-protein. Outbred New Zealand White rabbits were initially injected intradermally with 40 25- μg doses of purified BSP-binding protein in complete Freund's adjuvant. Animals were boosted intramuscularly with 500 μg of antigen in complete Freund's adjuvant after 2 mo and bled 7 and 9 d later. Subsequently, 7–9 d before each bleeding, an additional booster injection was given as above. The presence of antibody was monitored by double immunodiffusion (33) against the isolated BSP-binding protein. Purity of the antibody, and its reactivity to the bilirubin-binding protein, was also examined by double-radial immunodiffusion on agar plates with multiple peripheral wells, which contained up to six of the following antigens: the plasma membrane BSP- and bilirubin-binding proteins, a rat liver plasma membrane oleate-binding protein (29), rat serum albumin, ligandin, concentrated (ultrafiltered) rat bile, and the Triton X-100-solubilized mixture of LPM proteins. Monospecific antibody preparations were prepared by absorbing samples of immune rabbit serum with the concentrated membrane proteins not retained by the BSP-agarose affinity column (see above). IgG fractions of the antisera were prepared by standard techniques (36). In addition to preimmune rabbit serum, the IgG fraction of a rabbit anti-rat fibronectin was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego (CA) for use as a control antibody.

Effect of antibody on organic anion binding to LPM. Aliquots (0.4 mg) of the IgG fraction of the anti-BSP-binding protein or antifibronectin were incubated with varying quantities of LPM (0.625–10.0 mg membrane protein) in 1 ml PBS for 30 min at 37°C. Quantities of [^{14}C]bilirubin, [^{35}S]BSP, [^{14}C]taurocholate, and [^{14}C]oleate sufficient to saturate the incubated membrane aliquot were added in the form of a 1:4 complex in PBS with rat albumin, and incubated for an additional 30 min at room temperature. Membrane-bound radioactivity was determined as described above.

Immunohistochemical studies. The BSP-binding protein was localized in rat tissues by the indirect fluorescence antibody technique (37), using either of the two absorbed rabbit antisera against the BSP-binding-protein as the primary antibody, and a fluorescein-conjugated sheep anti-rabbit

gamma globulin (37) as the secondary probe. Acetone-fixed cryostat sections of snap frozen normal rat liver, kidney, and spleen, and unfixed viable hepatocytes in suspension, isolated mechanically (38) or by a collagenase technique (39) were used as substrates. Collagenase-isolated hepatocytes were kindly supplied by Dr. B. J. Potter and Dr. R. Nunes, Mount Sinai School of Medicine, New York. Additional studies were performed with rat livers obtained 4–5 d after bile duct ligation. Specificity of the immunohistochemical reaction in tissue sections and mechanically isolated hepatocytes was established by controls in which the primary antibodies were (a) first absorbed with either the purified membrane BSP-binding protein (1 mg/ml) at an antigen/antibody ratio of 2:1; rat serum albumin; or PBS containing 2.5 mg % BSP; or (b) replaced with either normal, nonimmune rabbit serum or PBS.

RESULTS

Characterization of LPM fractions. The isolated LPM fractions were similar to those utilized in previous studies (10, 29, 30), and were enriched 25-fold in 5'-nucleotidase and 12-fold in glucagon-stimulated adenylate cyclase compared with the initial homogenate (Table I). Contamination with microsomes or mitochondria was minimal, as assessed by both appropriate marker enzymes and electron microscopy. In randomly selected electron micrographs, a homogeneous picture of membrane structures containing numerous vesicles of various sizes was seen. Very few tight junctions, typical of canalicular membrane, were observed, but small amounts of rough endoplasmic reticulum were seen in many of the pictures. LPM protein recovery averaged 0.23 mg/g wet wt of liver.

Binding of organic anions to LPM. [^{14}C]Bilirubin, [^{35}S]BSP, [^{14}C]taurocholate, and [^{14}C]oleate each bound in saturable fashion to LPM (Fig. 1). The absolute quantities of each ligand bound varied somewhat with different batches of LPM, but results with any given batch were highly reproducible, as were the relative amounts of each ligand bound. Other than establishing the quantity of 1:4 ligand/albumin complex required to produce maximal binding of each test substance to LPM, as a reference point for antibody inhibition studies (see below), no attempt was made to further characterize ligand binding in this system.

Characterization of BSP- and bilirubin-binding LPM proteins. As previously reported (13), the protein recovered from bilirubin-agarose migrated as a single band with an estimated molecular mass of 55,000 D on SDS polyacrylamide gel electrophoresis, and cochromatographed with [^{35}S]BSP on Sephadex G-75. Yield of this protein was small, not exceeding 0.3% of the total protein applied to the affinity column.

Of the 500 mg membrane protein applied to the BSP-agarose column, $0.9 \pm 0.4\%$ (SD) was recovered after elution with 50 μM BSP. This protein also migrated as a single band with an estimated molecular

TABLE I
Activities of Marker Enzymes in Sinusoidal-enriched LPM Preparations
Compared with Initial Homogenate

Marker	Specific activity*		Purification† factor
	Homogenate	LPM	
5'-Nucleotidase	1.4±0.2§	34.4±2.8	24.3
Glucagon-stimulated adenylate cyclase	0.8±0.1	9.8±2.8	12.3
Glucose-6-phosphatase	4.1±0.7	1.4±0.4	2.9
Succinate dehydrogenase	1.04±0.2	0.17±0.03	6.1

* Micromoles (nanomoles for adenylate cyclase) substrate consumed · h⁻¹ · mg protein⁻¹.

† Ratio of LPM/homogenate specific activities for plasma membrane enzymes, or reciprocal for microsomal and mitochondrial enzymes.

§ All results mean±SD (n = 10).

mass of 55,000 D on SDS polyacrylamide gel electrophoresis (Fig. 2 A), and cochromatographed with [¹⁴C]bilirubin, as well as with [³⁵S]BSP, on Sephadex G-75. The higher yield, and disappearance of lower molecular mass contaminants observed in earlier studies (13), may reflect efforts made to block nonspecific binding to the affinity columns with ethanolamine.

When the BSP-binding protein was electrophoresed on polyacrylamide in the absence of SDS and without

dithiothreitol reduction, it again migrated as a single band (Fig. 2 B). An apparent molecular mass of 100,000 D was estimated by electrophoresis of the native protein on a polyacrylamide gradient gel (Fig. 2 C).

Gel filtration of the BSP-binding protein on a calibrated column of Sephadex G-150 (Figs. 3 and 4) resulted in elution of a single protein peak corresponding to a molecular mass of 103,000 D. If SDS was added to the sample before chromatography, two peaks, at 106,000 and 56,000, were eluted. Both peaks cochromatographed with [³⁵S]BSP.

The native dimeric BSP-binding protein had an isoelectric point of 3.5, and revealed positive staining with PAS. Mass spectrometry demonstrated the presence of sialic acid, at a ratio of 4 mol/mol of protein dimer. No lipid components were detectable with Sudan black. Absence of contamination with either albumin or ligandin was documented by radial immunodiffusion against antibodies to rat serum albumin and ligandin.

Characterization of rabbit antibodies against BSP-binding LPM protein. Whole immune sera from two rabbits repeatedly injected with BSP-binding protein produced a single precipitin line of identity between the purified bilirubin- and BSP-binding LPM proteins and the solubilized mixture of LPM proteins, but no reaction with rat ligandin, rat albumin, an oleate-binding protein isolated by affinity chromatography from LPM (29), or concentrated rat bile (Fig. 5). Some reactivity was also seen between whole rabbit immune sera and other, unidentified LPM components, but this was not observed with absorbed sera or with IgG fractions derived from them.

Antibody inhibition of organic anion binding to rat LPM. The purified antibody against the LPM BSP-binding protein inhibited binding of [³⁵S]BSP and [¹⁴C]bilirubin to LPM (Fig. 6). In incubations contain-

FIGURE 1 Binding of [¹⁴C]bilirubin, [³⁵S]BSP, [¹⁴C]taurocholate, and [¹⁴C]oleate to a typical rat LPM preparation. Each point represents the mean of triplicate determinations.

FIGURE 2 Polyacrylamide gel electrophoresis (PAGE) of the BSP-binding protein of rat LPM. (A, left panel) PAGE in SDS of the isolated protein after reduction with dithiothreitol. (B, center panel) PAGE of the unreduced protein without SDS. (C, right panel) Gradient PAGE of the unreduced protein without SDS. Molecular weight (MW) standards are also illustrated in panels A and C.

ing a constant amount of antibody (0.4 mg IgG fraction), a linear relationship was observed between the logarithm of the LPM concentration and the percent inhibition of binding, as determined by comparison with control incubations containing antifibronectin. At 0.6 mg LPM protein per incubation, the inhibition of [35 S]BSP binding was 80%, and of [14 C]bilirubin binding 65%. Binding of [14 C]oleate and [14 C]taurocholate under identical conditions was not affected. Control binding observed in the presence of antifibronectin was virtually identical to that observed in the presence of preimmune rabbit IgG.

Immunohistochemical studies. After incubation of normal rat liver sections with anti-BSP-binding protein, the plasma membrane of almost all hepatocytes was stained in a continuous linear or focally interrupted pattern (Fig. 7 A). In both tissue sections and isolated hepatocyte preparations (Fig. 7 B) all three domains of hepatocellular surface membranes appeared to bind antibody, although in some tissue specimens, the reaction with bile canaliculi was stronger than with sinusoidal and intercellular (lateral) membranes. Central veins; Kupffer, endothelial, and other sinusoidal cells; and portal tracts were not stained in

livers obtained from healthy rats. Rat livers obtained 4–5 d after ligation of the common bile duct showed a similar staining pattern of hepatocytes. In addition, the apical portion of proliferating bile ductular epithelial cells showed definite staining (Fig. 7 C). Absorption of the primary antibody with purified BSP-binding protein eliminated the staining of hepatocytes (Fig. 7 D), whereas absorption with rat serum albumin or PBS containing BSP had no effect. Replacement of the primary antibody by normal rabbit serum or PBS also abolished the staining. Normal rat kidney and spleen did not show any specific fluorescence.

DISCUSSION

At least three distinct classes of organic anions—fatty acids, bile acids, and nonbile acid cholephils such as BSP and bilirubin, are efficiently sequestered by the liver (1–3, 8, 40, 41) despite tight binding to albumin within the circulation (42–45). The mechanisms responsible for the hepatocellular uptake of these compounds are not well understood. Kinetic studies in a variety of systems suggest that BSP and bilirubin uptake may involve a common, membrane-associated

FIGURE 3 Cochromatography of the BSP-binding protein with [^{35}S]BSP on Sephadex G-150. In the absence of SDS, a single peak with an estimated molecular mass of 103,000 D is observed. In the presence of SDS, two peaks of 106,000 and 56,000 D are recovered. Both cochromatograph with [^{35}S]BSP. See text for experimental details.

carrier-mediated transport system (1-5), and that, despite some overlap (5-7), a separate system may be primarily responsible for bile acid uptake (1, 8, 9). The hepatocellular uptake mechanism for fatty acids is highly controversial, but at least some studies suggest that these, too, are sequestered by a saturable membrane-associated mechanism (46).

Since the first step in any membrane-associated carrier-mediated transport process involves binding to the carrier, several studies have focused on the binding of these anionic ligands to LPM, and attempts to identify and isolate putative receptors. The presence of a bile acid receptor in LPM preparations was proposed by Accatino and Simon (47) in 1974 on the basis of binding studies, and LPM proteins with high affinities for bile acids have subsequently been identified by photoaffinity labeling (48). More recently, specific binding

of oleate to LPM, and the isolation from LPM of a 40,000-D oleate-binding protein by affinity chromatography, have also been reported (29, 30).

The isolation of BSP- and bilirubin-binding proteins from rat LPM by affinity chromatography was first reported in 1977 (11). The protein isolated by its bilirubin affinity was shown to cochromatograph with [^{35}S]BSP, and both proteins had a molecular mass of $\sim 60,000$ D on SDS polyacrylamide gel electrophoresis (13). Although it was therefore considered likely that they represented the same protein, this presumption was not firmly established until the present studies, documenting that the BSP-binding protein also has a high affinity for bilirubin, and that the two proteins expressed immunologic identity as well. The BSP/bilirubin receptor protein is now recognized to be a $\sim 100,000$ -D dimer composed of two noncovalently

FIGURE 4 Calibration curve of the Sephadex G-150 column used for the studies illustrated in Fig. 3.

bound subunits of 56,000 D each, with an isoelectric point of 3.5, containing carbohydrate but no lipid compartments. Both the native protein and its subunits retain anion-binding properties. The presence of a common membrane receptor for both BSP and bili-

FIGURE 5 Agar double-immunodiffusion study. Absorbed rabbit antiserum to the rat LPM BSP-binding protein is in the center well. Peripheral wells contain (clockwise from top) (1) mixture of solubilized LPM proteins; (2) BSP-binding LPM protein; (3) bilirubin-binding LPM protein; (4) rat ligandin; (5) rat albumin; and (6) oleate-binding LPM protein. A line of identity is observed between the mixture of solubilized proteins and the BSP- and bilirubin-binding proteins. No antibody reactivity is seen with rat ligandin, albumin, oleate-binding protein or (in other studies not shown) the concentrated proteins of ultrafiltered rat bile.

FIGURE 6 Inhibition of the binding of [35 S]BSP and [14 C]bilirubin to LPM by rabbit antisera to the rat BSP-binding protein. In paired studies, varying quantities of LPM (0.625–10 mg protein) in 1 ml PBS were incubated for 30 min at 37°C with 0.4 mg IgG fraction of either the anti-BSP-binding protein or rabbit anti-rat fibronectin. Subsequently, 200 μ l of a solution of one of the ligands illustrated, as a 1:4 ligand/albumin complex, was added, and an additional incubation was carried out for 30 min at room temperature. In each case, sufficient ligand was added to saturate LPM under the conditions used. Binding of ligand to LPM was determined in the presence of each antibody, and results expressed as percent inhibition by the anti-BSP-binding protein, compared with binding observed in the presence of antifibronectin. Values are mean $\pm$ SD of at least five replicate experiments. BSP (X); bilirubin (O); oleate (●); taurocholate (□).

rubin explains the frequently reported mutual competitions between these two compounds for hepatic uptake (1–3, 49). Specific antibody-mediated inhibition of the binding of BSP and bilirubin, but not taurocholate or oleate, to LPM supports the hypothesis that a shared uptake mechanism exists for the former two compounds, which is distinct from the mechanisms that transport the latter.

Other groups have also reported the isolation of rat liver BSP- and bilirubin-binding proteins, using alternative methodologies. Wolkoff and Chung (14), in 1980, used photoaffinity labeling to identify the presence of such an LPM protein, estimated at 55,000 D by SDS-polyacrylamide gel electrophoresis, and designated it "organic anion-binding protein" (OABP). Although the reported isoelectric points of OABP and the BSP/bilirubin-binding protein described above differ, their similar anion-binding characteristics and migration on SDS polyacrylamide gel electrophoresis suggest that these are related, if not identical, proteins. Further evidence for similarity derives from their identical sialic acid content, the 4 mol/mol of protein dimer reported above corresponding to the 2 mol/mol

FIGURE 7 (A) Indirect immunofluorescent staining of hepatocytes in normal rat liver sections after incubation with rabbit antibody to BSP-binding protein. Sinusoidal, intercellular and canalicular surfaces of parenchymal cells all show specific staining. Kupffer cells, endothelial cells, and bile ductular cells are nonreactive. (B) Immunohistochemical staining of surface membranes of mechanically isolated rat hepatocytes, indicating presence of BSP-binding protein. (C) Staining of bile ductular epithelial cells, in addition to hepatocytes, observed in rat livers obtained 4-5 d after common bile duct ligation. (D) Inhibition of staining by prior absorption of the antibody with purified BSP-binding protein.

of 55,000-D subunit reported for OABP (14). Finally, in preliminary experiments, an antibody to OABP cross-reacted with a sample of the intact, 105,000-D BSP/bilirubin-binding protein.²

Tiribelli et al. (12) have described another BSP-binding protein, designated bilitranslocase. Initially isolated in the presence of deoxycholate, and reported

to have a molecular mass of 170,000 D, omission of deoxycholate has subsequently led to a lower molecular mass estimate of $\sim 100,000$ D (50). The latter protein can be resolved by reduction and SDS into two nonidentical subunits, α (molecular mass 37,000 D) and β (molecular mass 35,500 D). An overall subunit composition $\alpha_2\beta$ has been proposed (50). Any relationship between bilitranslocase and the BSP/bilirubin receptor protein described above remains to be established.

² Wolkoff, A. W., and W. Stremmel. Unpublished observations.

Previous data suggest that the affinities of BSP and bilirubin for the BSP/bilirubin receptor protein are greater than their corresponding affinities for albumin and ligandin (13). This study suggests that the protein is present on all three surface domains of the hepatocyte, but not in other cell populations within the normal liver, nor in kidney and spleen. All of these data point to a physiologic role for this protein in BSP and bilirubin transport. The significance of the unexpected and intriguing appearance of this protein on the luminal surface of obstructed bile ducts remains to be explored.

The precise contribution of the protein to organic anion transport remains to be determined, and several lines of investigation are indicated. Unfortunately, the obvious attempts to directly confirm its role in transport by antibody inhibition studies in both the isolated, perfused rat liver and in single cell suspensions of rat hepatocytes have been complicated by an apparent general cytotoxic effect of the antibodies thus far available. It is possible that this problem can be overcome by studies with isolated membrane vesicles (51). In any case, it is not clear whether the presence of high affinity organic anion receptors on hepatocyte plasma membranes can account for the unexpectedly rapid dissociation of anion/albumin complexes observed in vivo (1-3, 8, 40, 41), compared with dissociation rates observed in vitro (52-55). It has recently been proposed that this process is mediated by a separate albumin receptor (56, 57). This issue is unresolved (30, 58), but in view of the data presented above, confirmation of the existence of the albumin receptor would imply unique, two-receptor mechanisms for hepatocellular uptake of albumin-bound organic anions.

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REFERENCES

- Scharschmidt, B. F., J. G. Waggoner, and P. D. Berk. 1975. Hepatic organic anion uptake in the rat. *J. Clin. Invest.* **56**: 1280-1292.
- Goresky, C. A. 1977. Hepatic membrane carrier transport processes: their involvement in bilirubin uptake. In *The Chemistry and Physiology of Bile Pigments*. P. D. Berk and N. I. Berlin, editors, U. S. Government Printing Office, Wash. DC. 265-281.
- Paumgartner, G., and J. Reichen. 1976. Kinetics of hepatic uptake of unconjugated bilirubin. *Clin. Sci. Mol. Med.* **51**: 169-176.
- van Bezooijen, C. F. A., T. Grell, and D. L. Knook. 1976. Bromsulphophthalein uptake by isolated liver parenchymal cells. *Biochem. Biophys. Res. Commun.* **69**: 354-361.
- Laperche, Y., A.-M. Preaux, G. Feldmann, J.-L. Mahu, and P. Berthelot. 1981. Effect of fasting on organic anion uptake by isolated rat liver cells. *Hepatology*. **1**: 617-621.
- Mahu, J.-L., P. Duvaldestin, D. Dhumeaux, and P. Berthelot. 1977. Biliary transport of cholephilic dyes: evidence for two different pathways. *Am. J. Physiol.* **232**: E445-E450.
- Anwer, M. S., and D. Hegner. 1978. Effect of organic anions on bile acid uptake by isolated rat hepatocytes. *Hoppe-Seyler's Z. Physiol. Chem.* **359**: 1027-1030.
- Reichen, J., and G. Paumgartner. 1975. Kinetics of taurocholate uptake by the perfused rat liver. *Gastroenterology*. **68**: 132-136.
- Paumgartner, G., and J. Reichen. 1975. Different pathways for hepatic uptake of taurocholate and indocyanine green. *Experientia (Basel)*. **31**: 306-307.
- Reichen, J., B. L. Blitzer, and P. D. Berk. 1981. Binding of unconjugated and conjugated sulfobromophthalein to rat liver plasma membrane fractions in vitro. *Biochim. Biophys. Acta*. **640**: 298-312.
- Reichen, J., and P. D. Berk. 1977. Isolation of an organic anionic dye-binding protein from rat liver plasma membrane. *Gastroenterology*. **73**: 1242a. (Abstr.)
- Tiribelli, C., G. Lunazi, G. L. Luciani, E. Panfili, B. Gassin, G. Liut, G. Sandri, and G. Sottocasa. 1978. Isolation of a sulfobromophthalein-binding protein from hepatocyte plasma membrane. *Biochim. Biophys. Acta*. **532**: 105-112.
- Reichen, J., and P. D. Berk. 1979. Isolation of an organic anion-binding protein from rat liver plasma membrane fractions by affinity chromatography. *Biochem. Biophys. Res. Commun.* **91**: 484-489.
- Wolkoff, A. W., and C. T. Chung. 1980. Identification, purification, and partial characterization of an organic anion-binding protein from rat liver cell plasma membrane. *J. Clin. Invest.* **65**: 1152-1161.
- Barrett, P. V. D., F. X. Mullins, and N. I. Berlin. 1966. Studies on the biosynthetic production of bilirubin-C¹⁴: an improved method utilizing delta-aminolevulinic acid-4-¹⁴C in dogs. *J. Lab. Clin. Med.* **68**: 905-912.
- McDonagh, A. F. 1979. Bile pigments: bilatrienes and 5, 15 biladienes. In *The Porphyrins*. D. Dolphin, editor. Academic Press Inc., New York. 293-490.
- Heirwegh, K. P. M., J. Fevery, J. A. T. P. Meuwissen, and J. DeGroote. 1973. Recent advances in the separation and analysis of diazo-positive bile pigments. *Methods Biochem. Anal.* **22**: 205-250.
- Blanckaert, N., F. Compennolle, P. Leroy, L. R. Van Houtte, J. Fevery, and K. P. M. Heirwegh. 1978. The fate of bilirubin IX α glucuronide in cholestasis and during storage in vitro. *Biochem. J.* **171**: 203-214.
- Berk, P. D., C.-L. Kiang, W. Stremmel, and N. Tavoloni. 1982. A simple procedure for the isolation of bilirubin monoglucuronide and diglucuronide from bile. *J. Lab. Clin. Med.* **99**: 539-547.
- Bouner, W. M., and J. D. Stedman. 1978. Efficient fluorography of ³H and ¹⁴C on thin layers. *Anal. Biochem.* **89**: 247-256.
- Song, C. S., W. Rubin, A. B. Rifkind, and A. Kappas. 1969. Plasma membranes of the rat liver: isolation and

- enzymatic characterization of a fraction rich in bile canaliculi. *J. Cell Biol.* **41**: 124-132.
22. Fisher, M. M., D. L. Bloxam, M. Oda, M. J. Phillips, and Y. M. Yousef. 1975. Characterization of rat liver cell plasma membranes. *Proc. Soc. Exp. Biol. Med.* **150**: 177-184.
23. Lowry, O. H., N. J. Rosebrough, A. Lewis Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-279.
24. Baudhuin, P., P. Evrard, and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. *J. Cell Biol.* **32**: 181-191.
25. Michell, R. H., and J. N. Hawthorne. 1965. The site of diphosphoinositol synthesis in rat liver. *Biochem. Biophys. Res. Commun.* **21**: 333-338.
26. Wisher, M. H., and W. H. Evans. 1975. Functional polarity of the rat hepatocyte surface membrane: isolation and characterization of plasma-membrane subfractions from the blood-sinusoidal, bile-canalicular, and contiguous surfaces of the hepatocyte. *Biochem. J.* **146**: 375-388.
27. Nordlie, R. C., and W. J. Arion. 1966. Glucose-6-phosphatase. *Methods Enzymol.* **9**: 619-625.
28. Parkes, J. G., and W. Thompson. 1970. The composition of phospholipids in outer and inner mitochondrial membranes from guinea pig liver. *Biochem. Biophys. Acta.* **196**: 162-169.
29. Stremmel, W., S. Kochwa, and P. D. Berk. 1983. Studies of oleate binding to rat liver plasma membranes. *Biochem. Biophys. Res. Commun.* **112**: 88-95.
30. Stremmel, W., B. J. Potter, and P. D. Berk. 1983. Studies of albumin binding to rat liver plasma membranes: implications for the albumin receptor hypothesis. *Biochim. Biophys. Acta.* **756**: 20-27.
31. Porath, J., and L. Sundberg. 1972. High capacity chemisorbents for protein immobilization. *Nature New Biol.* **238**: 261-262.
32. Fehrstrom, H., and U. Moberg. SDS and conventional polyacrylamide gel electrophoresis with LKB 2117 Multiphor. Application Note 306, LKB, Broma, Sweden. 1-15.
33. Ouchterlony, O. 1958. Diffusion in gel methods. *Prog. Allergy.* **5**: 1-78.
34. Dewald, B., J. T. Dulaney, and O. Touster. 1974. Solubilization and polyacrylamide gel electrophoresis of membrane enzymes with detergents. *Methods Enzymol.* **32**: 82-91.
35. Roboz, J., R. Suzuki, and G. Bekesi. 1978. Determination of neuraminidase-susceptible and total N-acetylneuraminic acid in cells by selected ion monitoring. *Anal. Biochem.* **87**: 195-205.
36. Kochwa S. 1961. Isoagglutinins associated with ABO erythroblastosis. *J. Clin. Invest.* **40**: 874-883.
37. Gerber, M. A., E. Garfinkel, S. Z. Hirschman, S. N. Thung, and T. Panagiotatos. 1981. Immune and enzyme histochemical studies of a human hepatocellular carcinoma cell line producing hepatitis B surface antigen. *J. Immunol.* **126**: 1085-1089.
38. Hopf, U., K.-H. Meyer zum Buschenfelde, and M. P. Dierich. 1976. Demonstration of binding sites for IgG Fc and the third complement component (C3) on isolated hepatocytes. *J. Immunol.* **117**: 639-645.
39. Berry, N. M., and D. S. Friend. 1969. High yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* **43**: 506-520.
40. Glasinovic, J. C., M. Duval, and S. Erlinger. 1975. Hepatocellular uptake of taurocholate in the dog. *J. Clin. Invest.* **55**: 419-426.
41. Kushlan, M. C., J. Gollan, W.-L. Ma, and R. Ockner. 1981. Sex differences in hepatic uptake of long-chain fatty acids in single-pass perfused rat liver. *J. Lipid Res.* **22**: 431-436.
42. Goodman, D. S. 1958. The interaction of human serum albumin with long-chain fatty acid anions. *J. Am. Chem. Soc.* **80**: 3892-3898.
43. Baker, K. J., and S. E. Bradley. 1966. Binding of sulfo-bromophthalein (BSP) sodium by plasma albumin. Its role in hepatic BSP extraction. *J. Clin. Invest.* **45**: 281-287.
44. Ostrow, J. D., and R. Schmid. 1963. The protein-binding of ¹⁴C-bilirubin in human and murine serum. *J. Clin. Invest.* **42**: 1286-1298.
45. Kramer, W., H.-P. Buscher, K. Gerok, and G. Kurz. 1979. Bile salt binding to serum components. Taurocholate incorporation into high-density lipoprotein revealed by photoaffinity labelling. *J. Biochem.* **102**: 1-9.
46. Mahadevan, S., and F. Saver. 1979. Effect of trypsin, phospholipases, and membrane-impermeable reagents on the uptake of palmitic acids by isolated rat liver cells. *Arch. Biochem. Biophys.* **164**: 185-193.
47. Accatino, L., and F. R. Simon. 1976. Identification and characterization of a bile acid receptor in isolated liver surface membranes. *J. Clin. Invest.* **57**: 496-508.
48. Abberger, H., U. Bickel, H.-P. Buscher, K. Fuchte, W. Gerok, W. Kramer, and G. Kutz. 1980. Transport of bile acids: lipoproteins, membrane polypeptides, and cytosolic proteins as carriers. In *Bile Acids and Lipids*. G. Paumgartner, A. Stiehl, and W. Gerok, editors. MTP Press, Lancaster, England. 233-246.
49. Bloomer, J. R., and J. Zaccaria. 1976. Effect of graded bilirubin loads on bilirubin transported by perfused rat liver. *Am. J. Physiol.* **230**: 736-742.
50. Lunazzi, G., C. Tiribelli, B. Gazzin, and G. Sottocasa. 1982. Further studies on bilitranslocase, a plasma membrane protein involved in hepatic organic anion uptake. *Biochim. Biophys. Acta* **685**: 117-122.
51. Inoue, M., R. Kinne, T. Tran, and I. M. Arias. 1982. Taurocholate transport by rat liver sinusoidal membrane vesicles: evidence of sodium cotransport. *Hepatology.* **2**: 572-579.
52. Svenson, A., E. Holmer, and L. Andersson. 1974. A new method for the measurement of dissociation rates for complexes between small ligands and proteins as applied to the palmitate and bilirubin complexes with serum albumin. *Biochim. Biophys. Acta.* **342**: 54-59.
53. Faerch, T., and J. Jacobsen. 1975. Determination of association and dissociation rate constants for bilirubin-bovine serum albumin. *Arch. Biochem. Biophys.* **168**: 351-357.
54. Gray, R. D., and S. D. Stroupe. 1978. Kinetics and mechanism of bilirubin binding to human serum albumin. *Arch. Biochem. Biophys.* **168**: 351-357.
55. Scheider, W. 1978. Dissociation rate of serum albumin-fatty acid complex from stop-flow dielectric study of ligand exchange. *Biophys. J.* **24**: 260-262.
56. Weisiger, R., J. Gollan, and R. Ockner. 1981. Receptor for albumin on the liver cell surface may mediate uptake of fatty acids and other albumin bound substances. *Science (Wash. DC).* **211**: 1048-1051.
57. Weisiger, R., J. Gollan, and R. Ockner. 1982. The role of albumin in hepatic uptake processes. *Prog. Liver Dis.* **7**: 71-85.
58. Forker, E. L., and B. A. Luxon. 1981. Albumin helps mediate removal of taurocholate by rat liver. *J. Clin. Invest.* **67**: 1517-1522.