

# Identification, Purification, and Partial Characterization of an Organic Anion Binding Protein from Rat Liver Cell Plasma Membrane

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**ABSTRACT** Uptake of bilirubin, sulfobromophthalein (BSP), and other organic anions by the liver is a process with kinetics consistent with carrier mediation. The molecular basis of this transport mechanism is unknown. In the search for the putative organic anion carrier or receptor, the interaction of BSP with rat liver cell plasma membrane (LPM) has been studied. Specific binding of [<sup>35</sup>S]BSP to LPM was determined using a filtration assay. Results revealed high affinity ( $K_a = 0.27 \mu\text{M}^{-1}$ ), saturable (6.3 nmol/mg protein) binding, which was eliminated after preincubation with trypsin. Although [<sup>35</sup>S]BSP was strongly bound to LPM, the binding was rapidly reversible, preventing direct identification and study of a specific binding site(s). To avoid this problem, a photoaffinity probe was devised, in which [<sup>35</sup>S]BSP is covalently bound to LPM after exposure to ultraviolet light. Subsequent sodium dodecyl sulfate gel electrophoresis and fluorography revealed radioactivity predominantly associated with a single 55,000-mol wt protein. A protein with identical electrophoretic mobility was purified from deoxycholate solubilized LPM after affinity chromatography on glutathione-BSP-agarose gel. This protein migrated as a single band on sodium dodecyl sulfate gel electrophoresis and on urea gel isoelectric focusing. It contained 1–2 residues of sialic acid per 55,000-dalton protein, and was immunologically distinct from rat albumin and ligandin. It bound bilirubin with a  $K_d$  of 20  $\mu\text{M}$ , as determined by tryptophan fluorescence quenching. Although the high affinity of this LPM protein for organic anions suggests that it may function as a hepatocellular organic anion receptor, its role in transport of these compounds is unknown.

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## INTRODUCTION

Bilirubin is formed in reticuloendothelial cells, released into the circulation where it binds to albumin with high affinity, and subsequently excreted almost entirely by the liver (1, 2). After intravenous administration, sulfobromophthalein (BSP)<sup>1</sup> also binds strongly to albumin (3) and is excreted predominantly by the liver (2). The hepatic uptake of bilirubin and BSP is rapid. After the intravenous injection of tracer amounts of [<sup>3</sup>H]bilirubin or [<sup>35</sup>S]BSP in rats, ~40% of injected radioactivity is present within the liver 1.5 min later (2, 4–6). In the isolated perfused rat liver, 30–50% of an injected tracer bolus of [<sup>3</sup>H]bilirubin or [<sup>35</sup>S]BSP is removed in a single 40-s pass through the liver. If <sup>125</sup>I-albumin is injected simultaneously, all <sup>125</sup>I is recovered in the hepatic vein effluent confirming that albumin does not accompany bilirubin or BSP into the liver cell (7, 8).

The hepatic uptake of bilirubin and BSP is saturable (2, 9) and these compounds demonstrate mutual competition for uptake (2). When rats were preloaded with bilirubin, the plasma disappearance of a subsequent tracer dose of [<sup>3</sup>H]bilirubin was enhanced (2). Counter-transport of BSP and bilirubin (i.e., efflux of previously taken up radiolabeled ligand from liver after subsequent infusion with unlabeled ligand) has also been described (2), although these phenomena could simply represent efflux of ligand from intracellular binding sites. Bile acids do not compete with bilirubin and BSP for hepatic uptake (2, 9).

Based on these results, a facilitated carrier-mediated membrane transport mechanism is postulated (1, 2, 9) but its molecular basis is unknown. A role for ligandin, an abundant intrahepatocellular organic anion binding

<sup>1</sup>Abbreviations used in this paper: BSP, sulfobromophthalein; GSH, glutathione; LPM, liver cell plasma membrane; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

protein was postulated. However, hepatic ligandin concentration and the uptake rate constant for bilirubin did not correlate in studies of isolated perfused rat liver (7). The ligandin concentration correlated negatively with the efflux rate of bilirubin from liver into serum suggesting an intrahepatic sequestration or "storage" role for ligandin, rather than a direct role in transmembrane movement. Ligandin influences net hepatic uptake of organic anions by reducing their efflux from the hepatocyte after initial transfer from plasma into the cell (7).

The removal by the liver of other compounds including insulin (10), glucagon (11), and desialylated glycoproteins (12, 13) from plasma is dependent upon binding to specific plasma membrane receptors. The uptake of organic anions may also be mediated by a specific plasma membrane receptor. The binding of organic anions to purified liver cell plasma membrane (LPM) has been described (14–16), but its physiologic significance is unknown. In the present study, BSP binding to purified LPM is defined. Photoactivation of [<sup>35</sup>S]BSP after preincubation with LPM permitted identification of a membrane protein with which radioactivity was predominantly associated. This protein was purified to homogeneity by affinity chromatography on glutathione (GSH)-BSP-agarose and partially characterized.

## METHODS

**LPM preparations.** Male Sprague-Dawley rats (200–250 g) were obtained from Marland Farms (Peekskill, N. Y.) and fed ad libitum with Purina rat chow (Ralston Purina Co., St. Louis, Mo.). LPM was prepared from ~100 g of liver by zonal ultracentrifugation according to the method of Wisher and Evans (17) in a Beckman Ti 15 rotor (Beckman Instruments Inc., Fullerton, Calif.). The resulting crude plasma membrane preparation was subfractionated on a discontinuous sucrose gradient (17). As described by Wisher and Evans (17), three subfractions were obtained at sucrose densities of 1.13 (zonal-light), 1.16 (zonal-heavy A), and 1.18 (zonal-heavy B). These were stored at –80°C in 50 mM Tris-0.25 M sucrose, at pH 7.6 and used within 1 wk of preparation. 5' nucleotidase (18), glucose-6-phosphatase (19), bilirubin glucuronoside glucuronosyl transferase (20, 21), succinate dehydrogenase (22), and β-glucuronidase (23) activities were determined in the subfractions and compared to results in homogenate. Protein concentration was determined by the method of Lowry et al. (24), using bovine serum albumin as the standard. The specific binding of insulin to membrane subfractions was determined as described by Evans et al. (25) using porcine insulin (25.5 U/mg, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J.) and <sup>125</sup>I monoiodinated porcine insulin (100 μCi/μg, New England Nuclear, Boston, Mass.). The specific binding of glucagon to membrane subfractions was determined by the method of Desbouquois et al. (26) using unlabeled glucagon obtained from Schwarz/Mann Div., and <sup>125</sup>I monoiodinated glucagon (151.8 μCi/μg) obtained from New England Nuclear.

**Binding of [<sup>35</sup>S]BSP to LPM.** LPM (20–50 μg protein/20 μl) was incubated with 0.04–5.0 nmol (0.09–11.4 μM) of [<sup>35</sup>S]BSP (Amersham Corp., Arlington Heights, Ill. 40.3

mCi/mmol) in a polypropylene tube (Walter Sarstedt, Inc., Princeton, N. J.). The purity of [<sup>35</sup>S]BSP was >95% as judged by thin-layer chromatography (27). Except where noted, the reaction was performed in 20 mM phosphate buffer saline (PBS), pH 7.4, in a total volume of 0.44 ml. 1 min after incubation, 0.2 ml was rapidly vacuum filtered on a Whatman GF/C glass filter (Whatman Inc., Clifton, N. J.), which was quickly washed with 10 ml of ice-cold buffer. The filter was placed in a glass scintillation vial, 10 ml of Bray's solution (New England Nuclear) was added, and radioactivity was determined in an Intertechnique ABAC SL40 liquid scintillation counter (Intertechnique Instruments, Inc., Dover, N. J.) at a counting efficiency of 78%. Recovery of a given amount of <sup>35</sup>S was independent of the presence of a filter. The radioactivity of an unfiltered 0.2-ml aliquot of incubation medium was also determined. Binding of BSP to the filter was studied using 0.2 ml of [<sup>35</sup>S]-BSP (0.05–100 μM in PBS, pH 7.4). The optimal time of incubation (1–60 min) temperature (4–37°C), pH, and volume of filter wash (5–20 ml) were determined. To insure that the filters did not allow significant quantities of LPM to pass through, 20–50 μg of LPM protein was applied to a filter that was washed with 10 ml of PBS, pH 7.4. As a control, an identical aliquot of LPM was applied to another filter and placed directly into a test tube without washing. Protein content of both filters were determined and compared.

Nonspecific binding of [<sup>35</sup>S]BSP to LPM was defined as radioactivity which bound in spite of a 1,000-fold dilution of specific activity with unlabeled BSP. To measure specific and nonspecific binding, [<sup>35</sup>S]BSP (0.09–11.4 μM) was incubated with LPM in the presence and absence of a 1,000-fold molar excess of unlabeled BSP (0.09–11.4 mM). Radioactivity bound in the absence of excess unlabeled BSP was termed total binding, and radioactivity bound in the presence of excess unlabeled BSP was termed nonspecific binding. Specific binding represented the difference between total and nonspecific binding.

Binding of [<sup>35</sup>S]BSP to LPM was examined from the plot of  $\bar{v}$ /milligram protein vs. [F] where  $\bar{v}$  represents nmol of [<sup>35</sup>S]BSP bound per milligram of membrane protein and [F] is the molar concentration of free or unbound ligand (28). Because non-linearity of this plot of the data suggested more than one binding site, data were fit by computer using the least squares method of grids (29) to the equation representing two sets of binding sites with affinity constants  $K_{a1}$  and  $K_{a2}$  and capacity of  $n_1$  and  $n_2$ , moles of ligand per milligram membrane protein, respectively:

$$\bar{v} = \frac{n_1 K_{a1} [F]}{1 + K_{a1} [F]} + \frac{n_2 K_{a2} [F]}{1 + K_{a2} [F]} \quad (28)$$

To study reversibility of binding, 0.1 or 1.2 nmol of [<sup>35</sup>S]-BSP were incubated with 75 μg of LPM protein in a volume of 0.11 ml. 1 min later, 0.1 ml was either filtered and washed, or added to 10 ml of PBS, pH 7.4 with or without a 100-fold molar excess of unlabeled BSP. At 1, 2, 5, and 10 min after dilution, the entire 10-ml volume was rapidly filtered and washed with PBS, pH 7.4, and LPM bound radioactivity was determined. The effect of preincubation with trypsin was determined by incubation of 10 μl of a 2 mg/ml solution of trypsin (type III from bovine pancreas, Sigma Chemical Co., St. Louis, Mo.) in PBS, pH 7.4 with 0.2 ml of LPM (2–3 mg protein/ml) for 30 min at 37°C. Filter retention of LPM after trypsin pretreatment was determined by collecting the filtrate and wash of 300 μg of LPM protein. This was lyophilized and protein concentration determined. Results were compared to that of an identical amount of LPM at 4°C, to which an identical volume of trypsin was added immediately before filtration. The effect of preincubation with neuraminidase was

determined by incubation of a 5 U/ml solution of *Clostridium perfringens* neuraminidase (type IX, Sigma Chemical Co.) in PBS, pH 7.4 with 0.2 ml of LPM (2–3 mg protein/ml) for 30 min at 25°C (final neuraminidase concentration of 250 mU/ml). This concentration of neuraminidase was 50-fold that necessary to inactivate by desialylation over 90% of the plasma membrane receptor for asialoglycoproteins when the same neuraminidase preparation was used.<sup>2</sup> The effect of taurocholate (Sigma Chemical Co.) on [<sup>35</sup>S]BSP binding to LPM was studied by incubating sodium taurocholate (final concentration 0.3 mM) with LPM and [<sup>35</sup>S]BSP in PBS, pH 7.4, and determining bound BSP. A control incubation lacking taurocholic acid was performed at the same time. Rat erythrocyte ghosts were prepared by the method of Dodge and Mitchell (30), and binding of [<sup>35</sup>S]BSP was studied in a manner identical to that used for study of LPM. Because GSH-BSP was used as an affinity adsorbent in affinity chromatography studies of LPM, competition of this compound with [<sup>35</sup>S]BSP for binding to LPM was examined. These studies were performed identically to the binding studies described above, except for inclusion of 22 μM GSH-BSP in the incubation mixture.

**Photoaffinity probe of BSP binding to LPM.** Although BSP was strongly bound to LPM, the binding was rapidly reversible, which prevented direct identification and study of a specific binding site(s). To avoid this problem, a photoaffinity probe was devised, in which [<sup>35</sup>S]BSP is covalently bound to LPM after exposure to ultraviolet (UV) light. 0.2 ml of each of the LPM subfractions (2–3 mg protein/ml), in 50 mM Tris-0.25 M sucrose, pH 7.6 was pipetted into a 5-ml plastic beaker containing a stirring bar. This beaker was fit into a larger beaker containing ice and the two beakers were placed on a magnetic stirrer. [<sup>35</sup>S]BSP (0.5–1.0 nmol/mg protein) in 0.02 ml was added and allowed to equilibrate for 1 min. The mixture was irradiated from above with short wave (254 nm) UV light (Ultra-Violet Products, Inc., San Gabriel, Calif. model R-52, 1,300 W/cm<sup>2</sup> at 15 cm) for 3 min. The same amount of [<sup>35</sup>S]BSP was again added, and the procedure repeated for an additional 3 min. Sodium dodecyl sulfate (SDS) slab gel electrophoresis of the modified LPM was performed in 10% polyacrylamide (Bio-Rad Laboratories, Richmond Calif.) with a 3% spacer gel using Tris-glycine running buffer (31). SDS (Gallard-Schlesinger) was recrystallized from absolute ethanol (32). After electrophoresis, the gel was stained for 30 min with Coomassie Brilliant Blue (0.2%), and destained with ethanol/acetic acid/water (5:7.5:87.5). The gel was dehydrated in dimethyl sulfoxide and impregnated with 2,5-diphenyloxazole (New England Nuclear) before drying (33). Radioactivity was localized by fluorography using preflashed Kodak SB-54 film (34) (Eastman Kodak Co., Rochester, N. Y.).

**GSH-BSP-agarose affinity chromatography of solubilized LPM.** Identification of a specific LPM protein that bound photoactivated BSP suggested that this protein could be isolated by affinity chromatography. GSH-BSP was synthesized (35) and coupled to cyanogen bromide-activated agarose (Sephacrose 4B) (31). The zonal-heavy A and zonal-heavy B LPM subfractions (2–3 mg protein/ml) were mixed with sodium deoxycholate (0.4% final vol) and allowed to stand on ice for 30 min. After centrifugation for 20 min at 10,000 g, the supernate was charged onto a 25 ml GSH-BSP-agarose affinity column. The column was washed with 120 ml of PBS, pH 7.4 and eluted with 0.01 M Tris, pH 8.8 (4°C). At this pH, binding of [<sup>35</sup>S]BSP was reduced as compared to that at pH 7.4 (Fig. 2). Fractions were collected in 3-ml aliquots, and absorption at 280 nm indicated protein concentration. Protein-

containing fractions, which eluted at pH 8.8, were combined. The solution was concentrated to a final volume of 5 ml in an Amicon model 12 stirred cell with a PM 10 filter (Amicon Corp., Lexington, Mass.). The resulting protein solution was lyophilized and stored at –80°C.

The protein composition of the eluate was studied by SDS slab gel electrophoresis on a 10% polyacrylamide gel (31); less than 0.1 μg of protein could easily be detected in a single band. Isoelectric focusing was performed in 6 M urea (36). Sialic acid content was determined by the method of Warren (37). Amino acid analysis was performed after hydrolysis for 24 h in 6 N HCl at 105°C using a Technicon TSM amino acid autoanalyzer (38) (Technicon Instruments Corp., Tarrytown, N. Y.). Immunodiffusion studies were performed in agar gel and reactivity of the concentrated protein (0.1 ng/ml) against monospecific antibodies to rat albumin and ligandin was tested (39). The isolated protein was iodinated by the chloramine T method (40), using Na<sup>125</sup>I obtained from New England Nuclear (17 Ci/mg). Distribution of radioactivity after SDS slab gel electrophoresis of the iodinated protein was determined by radioautography using Kodak XR-5 film and a polyester base high speed image intensification screen (Picker Corp., Cleveland, Ohio). In 4 h at –70°C, <1,000 cpm of <sup>125</sup>I could easily be detected.

The binding of bilirubin to the isolated protein was determined by measuring the decrease in intrinsic fluorescence of protein upon addition of ligand (4, 41). A Perkin-Elmer fluorescence spectrometer MPF-3 (Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.) with cuvettes of 0.5 ml total volume was used. The cuvettes contained 0.6 μM protein in 0.15 M Tris, pH 8.2, in 0.4 ml. The temperature was maintained at 25°C. Bilirubin was dissolved in 0.05 N NaOH in a final concentration of 0.3 mM, and was immediately added to the cuvette in 0.002 ml increments to generate fluorescence changes, which were measured at 330 nm after excitation at 285 nm. The absorbance of bilirubin was sufficiently low that correction for inner filter effects (42) was unnecessary, and addition of diluent alone did not result in a decrement in fluorescence. Bilirubin did not fluoresce at the wavelengths studied. After correction for dilution, a reciprocal plot of change in fluorescence vs. bilirubin concentration was evaluated by least squares linear regression. Dissociation constants were calculated as the negative reciprocal of the x-intercept (4, 41). Because BSP absorbs light at 330 nm, its binding could not be studied by this method.

## RESULTS

**LPM preparation.** Recovery of protein and 5'-nucleotidase activity in the LPM preparation is seen in Table I. The zonal-light subfraction was enriched in 5' nucleotidase and bilirubin glucuronoside glucuronosyl transferase activities as compared to the zonal-heavy A and heavy B subfractions. Specific activities of glucose-6-phosphatase, β-glucuronidase, and succinate dehydrogenase were reduced as compared to specific activities in the homogenate (Table I). Specific binding of insulin and glucagon was 3–5 times higher in the zonal heavy subfractions than in the zonal-light subfraction (Fig. 1).

**Binding of [<sup>35</sup>S]BSP to LPM.** In the absence of LPM, <0.2% of [<sup>35</sup>S]BSP bound to the filters. In comparison to the amount of [<sup>35</sup>S]BSP bound in the presence of LPM, these counts were negligible, and were

<sup>2</sup> A. G. Morell and R. J. Stockert. Personal communication.

TABLE I  
Protein Content and Marker Enzyme Activities in Liver Homogenate and LPM Subfractions

Protein	5' nucleotidase	Bilirubin glucuronoside glucuronosyl transferase	Glucose-6-phosphatase	$\beta$ -glucuronidase	Succinate dehydrogenase	
mg/g liver	$\mu$ mol inorganic phosphorous released/mg protein/20 min	nmol bilirubin diglucuronide formed/min/mg protein	$\mu$ mol inorganic phosphorous released/mg protein/20 min	nmol phenolphthalein glucuronide hydrolyzed/mg protein/60 min	nmol succinate reduced/mg protein/min	
Homogenate	184 $\pm$ 7.9 (16)	1.28 $\pm$ 0.09(16)	0.57 $\pm$ 0.09(3)	0.25 $\pm$ 0.06(3)	0.88(1)	5.3(1)
Zonal-light	0.018 $\pm$ 0.003 (16)	78.6 $\pm$ 8.4 (16)	20.8 $\pm$ 6.6 (3)	<0.006(3)	0.36(1)	<0.003(1)
Zonal-heavy A	0.060 $\pm$ 0.012(16)	8.9 $\pm$ 1.2 (16)	12.2 $\pm$ 6.4 (3)	<0.005(3)	0.08(1)	<0.001(1)
Zonal-heavy B	0.080 $\pm$ 0.011(16)	4.2 $\pm$ 0.7 (16)	5.7 $\pm$ 2.7 (3)	<0.004(3)	0.08(1)	<0.001(1)

not corrected for in later calculations. The filters retained over 95% of applied LPM protein. After pretreatment of LPM with trypsin, they retained over 90% of LPM protein. The association of [<sup>35</sup>S]BSP with LPM was rapid; maximal binding was attained by 1 min, which was used in subsequent studies. Washing the filter with 20 ml of buffer did not remove more radioactivity than did a 10-ml wash, and a 10-ml wash was used in subsequent studies. Binding remained constant between 4° and 37°C, and there was no change in binding of [<sup>35</sup>S]BSP during the time that LPM was stored frozen. Binding of [<sup>35</sup>S]BSP was relatively constant between pH 6.5 and 8.0 (Fig. 2). Binding decreased above pH 7.4, and increased below pH 6.5. Because of the unusual shape of this binding curve, an additional study was performed. Pretreatment of LPM with trypsin did not reduce binding of [<sup>35</sup>S]BSP

as compared to intact LPM when studied at pH 3.5; at pH 7.4, binding was reduced by 90%. These different binding characteristics suggest differences in the mechanisms of ligand-LPM interaction at these pH values. It is possible that at low pH, the now largely nonionized BSP molecule binds preferentially to membrane lipids. Subsequent studies were performed at the more physiologic pH 7.4, in 20 mM PBS.

*Characteristics of [<sup>35</sup>S]BSP binding to LPM.* In the presence of 1,000-fold excess of unlabeled BSP, non-specific binding of [<sup>35</sup>S]BSP to LPM was <3% of total binding, and was not corrected for in subsequent studies. Binding of [<sup>35</sup>S]BSP to LPM was rapidly reversible upon dilution 100-fold with buffer; 80% of LPM-bound [<sup>35</sup>S]BSP dissociated within 1 min. No

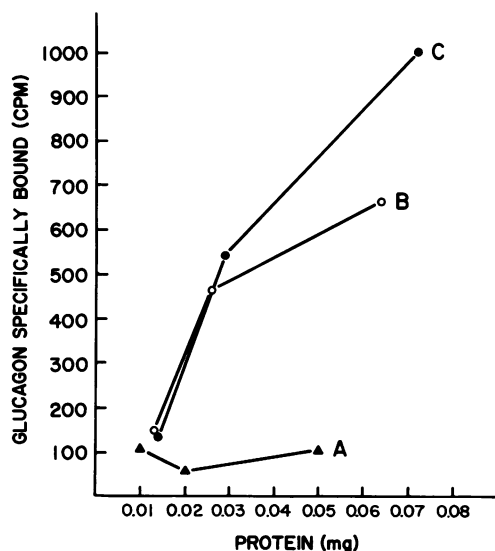


FIGURE 1 Specific binding of <sup>125</sup>I-glucagon to the zonal-light (A), zonal-heavy A (C), and zonal-heavy B (B) LPM subfractions. Experimental details are as in the text.

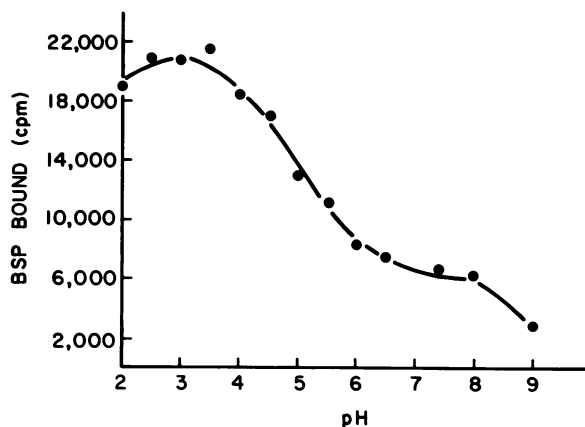


FIGURE 2 Effect of pH on binding of [<sup>35</sup>S]BSP to LPM. In this study, 61,000 cpm (1.4 nmol) of [<sup>35</sup>S]BSP in 0.01 ml was incubated with 0.02 ml of zonal-heavy B LPM and 0.2 ml of 0.15 M sodium phosphate buffer at pH 2-9; the final concentration of [<sup>35</sup>S]BSP was 6.2  $\mu$ M. LPM bound radioactivity was determined as in the text. Pretreatment of LPM with trypsin did not reduce binding of [<sup>35</sup>S]BSP as compared to intact LPM when studied at pH 3.5; at pH 7.4, binding was reduced by 90%. Similar results were obtained with the zonal-light and heavy A subfractions, using [<sup>35</sup>S]BSP concentrations as low as 0.3  $\mu$ M.

change in the rate of dissociation was seen when dilution was performed in the presence of a 100-fold molar excess of unlabeled BSP (Fig. 3). This suggested that there was no significant negative cooperativity (43).

All three LPM subfractions bound [<sup>35</sup>S]BSP. Scatchard plots of the concentration of bound ligand per milligram membrane protein vs. the ratio of bound to free ligand per milligram membrane protein, were non-linear. Because there was no evidence for negative cooperativity, this suggested high and low affinity binding sites (28). Computer analysis revealed that the light subfraction had a high affinity site, which bound [<sup>35</sup>S]BSP with  $K_{a1}$ , of  $0.54 \mu\text{M}^{-1}$  and  $n_1$  of 2.6 nmol/mg protein, and a low affinity site with  $K_{a2} = 0.01 \mu\text{M}^{-1}$  and  $n_2 = 27 \text{ nmol/mg protein}$ . The heavy subfractions had  $K_{a1}$ , of  $0.27 \mu\text{M}^{-1}$  and  $n_1$  of 6.3 nmol/mg protein with  $K_{a2}$  of  $0.01 \mu\text{M}^{-1}$  and  $n_2$  of 31 nmol/mg protein (Fig. 4). Preincubation of all three LPM subfractions with trypsin virtually eliminated binding (Fig. 5). Preincubation with neuraminidase had no effect on binding of [<sup>35</sup>S]BSP by LPM. Taurocholic acid in a concentration as high as 0.3 mM had no effect on binding of [<sup>35</sup>S]BSP to LPM. GSH-BSP competitively inhibited binding of [<sup>35</sup>S]BSP to all three subfractions with an inhibition constant of  $10 \mu\text{M}$  for the high affinity binding site. Unlike the case for LPM, binding of [<sup>35</sup>S]-BSP by rat erythrocyte ghosts generated a linear Scatchard plot, typical of a single class of binding sites. Analysis revealed  $K_a$  of  $0.023 \mu\text{M}^{-1}$  with 15.2 nmol of [<sup>35</sup>S]BSP bound per milligram of protein. These results are

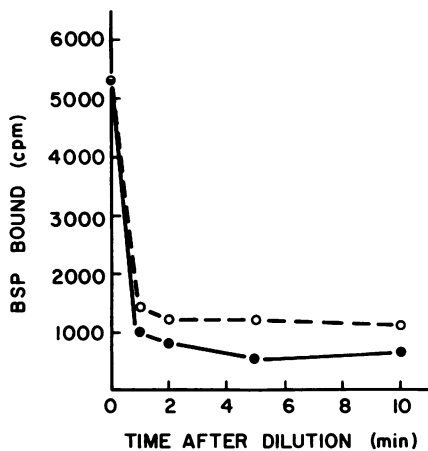


FIGURE 3 Reversibility of binding of [<sup>35</sup>S]BSP to LPM. Experimental details are as in the text. 1 min after incubation of [<sup>35</sup>S]BSP with LPM, 0.1 ml of the mixture was either filtered and washed to determine bound BSP, or diluted 100-fold with PBS (O) or with PBS containing a 100-fold molar excess of unlabeled BSP (●). At intervals after dilution, the entire volume was filtered and bound radioactivity was quantitated. This experiment was performed with the zonal-heavy B subfraction; similar results were obtained for the other two LPM subfractions.

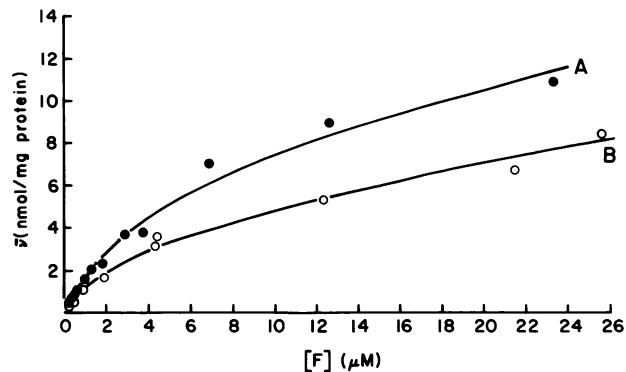


FIGURE 4 Plot of bound BSP ( $\bar{v}$ ) vs. the concentration of free BSP [F] as determined for the zonal-heavy B (A) and zonal-light (B) subfractions. Results for the zonal-heavy A subfraction were identical to those for the zonal-heavy B subfraction. For each LPM subfraction, binding was analyzed, as in the text, to the equation representing two sets of binding sites with affinity constants  $K_{a1}$  and  $K_{a2}$ , and capacity of  $n_1$  and  $n_2$  nanomoles of ligand per milligram membrane protein. The lines represent least squares fits to the data. The zonal-light subfraction had  $K_{a1} = 0.54 \mu\text{M}^{-1}$ ,  $K_{a2} = 0.01 \mu\text{M}^{-1}$ ,  $n_1 = 2.6 \text{ nmol/mg protein}$ , and  $n_2 = 27 \text{ nmol/mg protein}$ . The zonal heavy subfractions had  $K_{a1} = 0.27 \mu\text{M}^{-1}$ ,  $K_{a2} 0.01 \mu\text{M}^{-1}$ ,  $n_1 = 6.3 \text{ nmol/mg protein}$ , and  $n_2 = 31 \text{ nmol/mg protein}$ .

similar to binding of [<sup>35</sup>S]BSP by the low affinity binding site of LPM.

*Photoaffinity probe of BSP binding to LPM.* SDS gel electrophoresis of the two zonal-heavy subfractions revealed almost identical patterns, which differed from that of the zonal-light subfraction (Fig. 6). LPM from the zonal-heavy subfractions was incubated with 0.5 nmol of [<sup>35</sup>S]BSP/mg protein, irradiated with UV light, and SDS gel electrophoresis was performed. Fluorography of the gel revealed radioactivity predominantly associ-

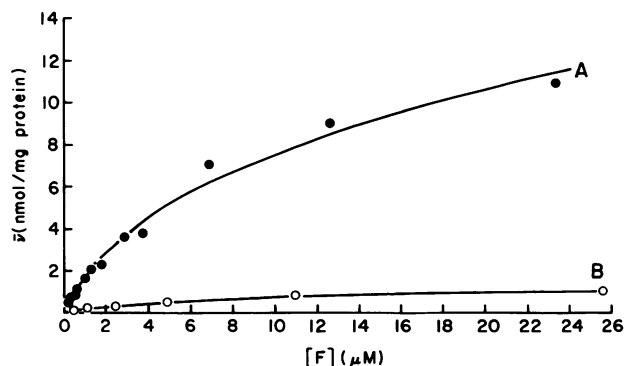


FIGURE 5 Effects of trypsin preincubation on LPM binding of [<sup>35</sup>S]BSP. LPM was incubated for 30 min at 37°C with (B) and without (A) 100  $\mu\text{g/ml}$  of trypsin. Binding of [<sup>35</sup>S]BSP to LPM was virtually eliminated following trypsin preincubation. Results in this illustration are for the zonal-heavy B subfraction, but were identical to results for the other two subfractions.

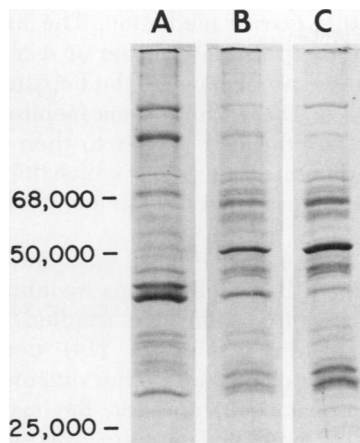


FIGURE 6 SDS polyacrylamide (10%) slab gel electrophoresis of the zonal-light (A), zonal-heavy A (B), and zonal-heavy B (C) LPM subfractions. Samples were solubilized in 0.4% SDS with 1%  $\beta$ -mercaptoethanol, and  $\sim 20 \mu\text{g}$  of membrane protein was applied to the gel. Positions of bovine serum albumin (68,000 mol wt) and gamma globulin subunits of 50,000 and 25,000 mol wt are indicated at the left.

ated with a single protein band of mol wt  $\sim 55,000$  (Fig. 7). When the same procedure was performed without irradiation, no protein-associated radioactivity was detected. With larger amounts of  $[^{35}\text{S}]\text{BSP}$ , radioactivity was nonselectively associated with many proteins.

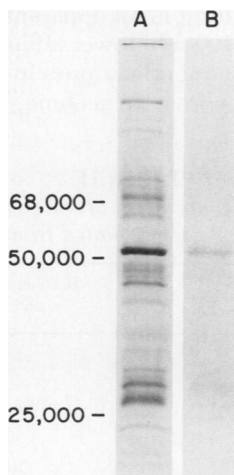


FIGURE 7 Photoaffinity probe of  $[^{35}\text{S}]\text{BSP}$  to LPM subfraction zonal-heavy B. A Coomassie Blue stained 10% polyacrylamide slab gel of this LPM subfraction is in column A. The adjacent lane contained LPM to which  $[^{35}\text{S}]\text{BSP}$  had been attached as in the text; its fluorogram is in column B. Solubilization of samples and mol wt markers are as described in Fig. 6. Radioactivity is predominantly associated with a protein of  $\sim 55,000$  mol wt. Identical results were obtained with the zonal-heavy A subfraction. Similar study of the zonal-light subfraction revealed association of radioactivity with three protein bands of 20,000, 23,000, and 40,000 daltons, with no radioactivity in the 55,000-mol wt region.

Fluorography of zonal-light LPM, which had been incubated with a similar amount of  $[^{35}\text{S}]\text{BSP}$  and irradiated with UV light, revealed association of radioactivity with three protein bands (20,000, 23,000, and 40,000 daltons) with no single predominant band. There was no radioactivity in the 55,000-mol wt region.

**Affinity chromatography of solubilized LPM.** Over 90% of LPM protein was present in the supernate after solubilization of the zonal-heavy A or zonal-heavy B subfractions with 0.4% deoxycholate. Washing the column with PBS, pH 7.4, removed 70–80% of applied protein. Elution with 10 mM Tris, pH 8.8 (4°C) removed a peak containing 1–2% of applied protein (Fig. 8). SDS gel electrophoresis of as much as  $10 \mu\text{g}$  of protein from this peak revealed a single protein band of 55,000 daltons (Fig. 9), which migrated in the same position as did radioactivity when fluorography was performed. After iodination of this protein with  $^{125}\text{I}$ ,  $\sim 100,000$  cpm were subjected to slab gel electrophoresis on 10% polyacrylamide. Radioautography of the gel revealed that all radioactivity was in a single band corresponding to a mol wt of 55,000. Identical results were obtained when extracts of either of the two zonal-heavy subfractionations were chromatographed. Because of a relatively poor yield on subfractionation of LPM, studies of affinity chromatography of the zonal-light subfraction have not, as yet, been performed.

Isoelectric focusing of up to  $5 \mu\text{g}$  of the purified protein was performed in 6 M urea. Without urea, the protein precipitated at the top of the gel and did not enter. After staining with Coomassie Brilliant Blue, a single protein band was found (Fig. 10). Amino acid analysis revealed a relative enrichment in phenylalanine and acidic residues (Table II). There were 1–2

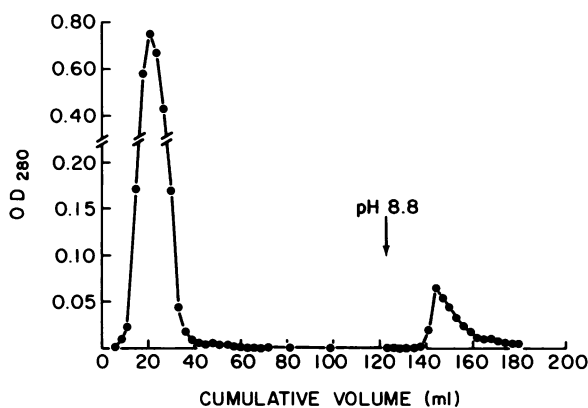


FIGURE 8 Affinity chromatography of deoxycholate solubilized LPM on GSH-BSP-agarose gel. LPM was solubilized in 0.4% deoxycholate, centrifuged for 20 min at 10,000 g, and the supernate was charged onto a 25 ml GSH-BSP-agarose gel affinity column. The column was washed with 120 ml of PBS, pH 7.4 and eluted with 0.01 M Tris, pH 8.8 (4°C). Absorption at 280 nm indicated protein concentration.

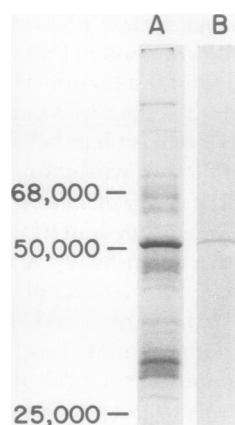


FIGURE 9 SDS polyacrylamide (10%) slab gel electrophoresis patterns of approximately 25  $\mu\text{g}$  of the zonal-heavy B LPM subfraction (A) and 5  $\mu\text{g}$  of the isolated organic anion binding protein (B) after staining with Coomassie Blue. Solubilization of samples and molecular weight markers are as in Fig. 6. The isolated protein migrates as a single band of  $\sim 55,000$  mol wt. Identical results were obtained for the zonal-heavy A subfraction.

residues of sialic acid/55,000-dalton protein. Diffusion of purified protein against monospecific antibody to rat albumin and rat ligandin revealed no immunoreactivity. Studies of the binding of bilirubin to the isolated protein revealed a dissociation constant of 20  $\mu\text{M}$  (Fig. 11).

## DISCUSSION

The uptake of bilirubin, BSP, and other organic anions by the liver is rapid and has kinetic character-

istics suggesting carrier mediation. The biochemical mechanism of the presumed carrier or receptor is unknown. As these anions traverse the hepatic sinusoids into the space of Disse, the plasma membrane of the hepatocyte offers the first barrier to their entry into the cell. Whether this is the site at which the specificity and carrier-mediated kinetics of anion uptake occur is not known.

In an attempt to answer this question, the interaction of isolated liver cell plasma membrane preparations with organic anions was studied by several investigators. Cornelius et al. (14) demonstrated saturable binding of BSP and other organic anions to a rat LPM preparation; however, the magnitude of binding ( $\sim 200$  nmol/mg protein) was larger than expected for a physiologic process. Bilirubin competes for uptake of BSP by the liver in vivo, but had no effect on binding in vitro. Reichen et al. (16) determined  $K_d$  of BSP binding to LPM as 390–650  $\mu\text{M}$ , with saturation at 230–440 nmol/mg protein. Although results of these two studies are in agreement, the number of binding sites is high, and the affinity of BSP for LPM is lower by two orders of magnitude than is the affinity of BSP for albumin (3), from which it is extracted before entering the hepatocyte. Tiribelli et al. (15) studied BSP binding to rat LPM by continuous flow dialysis. High affinity binding of BSP was described with  $K_d$  of 4.88  $\mu\text{M}$  and saturation at 40.4 nmol BSP/mg protein. Bilirubin (0.5 mM) competitively inhibited BSP binding, resulting in an apparent  $K_d$  of 10.5  $\mu\text{M}$ , which indicates  $\sim 100$ -fold lower affinity of membrane for bilirubin. Subsequently, a protein was isolated by gel chromatography from an acetone powder of crude

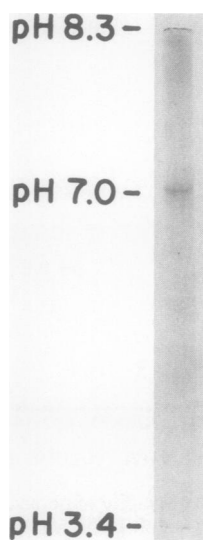


FIGURE 10 Isoelectric focusing gel of  $\sim 3 \mu\text{g}$  of the isolated organic anion binding protein after staining with Coomassie Blue. This procedure was performed in 6 M urea and reveals a single protein band of isoelectric point 7.

TABLE II  
Amino Acid Composition of the Organic Anion Binding Protein Isolated from LPM

Amino acid	Residues/1,000 amino acid residues
Aspartic acid	86
Methionine	14
Threonine	53
Serine	64
Glutamic acid	124
Proline	59
Glycine	96
Alanine	88
Valine	69
Isoleucine	58
Leucine	93
Tyrosine	17
Phenylalanine	40
Lysine	63
Histidine	26
Arginine	49

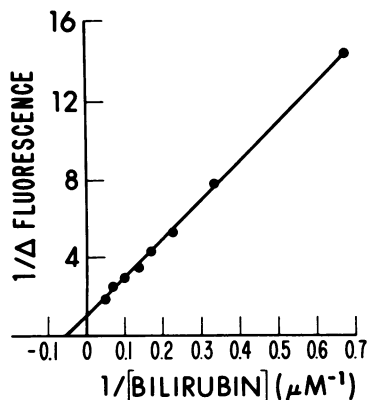


FIGURE 11 Binding of bilirubin to the isolated organic anion binding protein. Details are as in the text. A double reciprocal plot of the decrement in fluorescence of the protein vs. the concentration of added ligand is shown. The dissociation constant ( $20 \mu\text{M}$ ) is calculated as the negative reciprocal of the  $x$ -intercept.

LPM (44). This 170,000-dalton protein binds over 100 nmol of BSP per mg, implying at least 17 binding sites for BSP;  $K_d$  was  $4 \mu\text{M}$ . Whether the large number of binding sites indicates unusual binding kinetics or is an artifact of the methodology is not clear.

In the present study of BSP binding to LPM subfractions, high and low affinity binding sites were detected.  $K_a$  of the high affinity site is close to that described by Tiribelli et al. differs from results in the two previous studies and is similar to  $K_a$  of BSP for rat serum albumin (3) or rat ligandin (45). The strong affinity of BSP for LPM suggests that this binding may represent a physiologic process.

The virtual elimination of BSP binding to zonal-heavy and light LPM after preincubation with trypsin suggests that BSP interacts with a LPM protein(s). To identify the BSP binding site, [ $^{35}\text{S}$ ]BSP was photoactivated after preincubation with LPM. In studies of zonal-heavy LPM, when [ $^{35}\text{S}$ ]BSP was present in low concentration, radioactivity was associated predominantly with a single 55,000-mol wt protein after SDS gel electrophoresis of the zonal-heavy A or B subfractions. Other proteins were accessible to [ $^{35}\text{S}$ ]BSP as demonstrated by the fact that irradiation of LPM after preincubation with a high concentration of [ $^{35}\text{S}$ ]BSP resulted in association of radioactivity with many proteins. A similar study performed using the zonal-light subfraction revealed binding to three protein bands (20,000, 23,000, and 40,000 daltons) with no single predominant band of radioactivity and no radioactivity in the 55,000-mol wt region. Whether these bands represent distinct proteins or are components of a single protein is not known, and the high affinity binding site for BSP may be on either or all of these proteins. These photoaffinity studies suggest that the similar binding kinetics of [ $^{35}\text{S}$ ]BSP to zonal-heavy

and light LPM may be due to binding of ligand to different proteins of comparable affinities. Wisher and Evans (17) suggest that the two heavy subfractions are derived from the sinusoidal and contiguous faces of the hepatocyte, whereas the light subfraction is derived from the canalicular aspect. If this localization of subfractions is correct, the 55,000-mol wt binding protein would be in the anticipated position to function in hepatic uptake of organic anions. To purify this protein, affinity chromatography of deoxycholate solubilized LPM was performed on GSH-BSP-agarose gel. Purification was demonstrated by migration of the protein as a single 55,000-mol wt band after SDS gel electrophoresis.

Results of biochemical and electron microscopic studies suggest that the organic anion binding protein is a component of liver cell plasma membranes. Because it is uncertain whether any biochemical markers are specific for only one surface of the hepatocyte, the sinusoidal localization of these two zonal-heavy subfractions is tentative. Biochemical studies (Table I) reveal that they are enriched in the plasma membrane markers 5'-nucleotidase and bilirubin glucuronoside glucuronosyl transferase, as compared to homogenates. Activities of glucose-6-phosphatase (endoplasmic reticulum),  $\beta$ -glucuronidase (lysosomes), and succinate dehydrogenase (mitochondria) are  $<10\%$  of that in homogenate. Mr. Jonathan Rothblatt, in Dr. Alex B. Novikoff's laboratory (Albert Einstein College of Medicine, Bronx, N. Y.), examined a pelleted aliquot of the zonal-heavy A subfraction through its entire depth, at a magnification of 1,300, and random areas were viewed at 10,000. No nuclei, mitochondria, rough endoplasmic reticulum or other recognizable organelles contaminated the fraction, save for a single residual body. The origin of the membranes could not be established, but recognizable bile canalicular fronts were not seen.

BSP adsorbs light at 330 nm and binding to the isolated protein could not be determined by the tryptophan fluorescence quenching method. Because the isolated protein precipitates and binds to dialysis tubing, equilibrium dialysis could not be used to study BSP binding. By contrast, bilirubin bound to the isolated protein with high affinity. Although the fluorography experiments indicate that the protein binds [ $^{35}\text{S}$ ]BSP in isolated LPM, its potential role in vivo is speculative. Its role as a liver cell plasma membrane receptor for organic anions, and relationship to the intracellular transport of these compounds is unknown. Identification of animal models in which the hepatic uptake of organic anions is reduced, such as mutant Southdown sheep and fetal or neonatal animals, may elucidate the relationship of this putative receptor to hepatic transport.



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