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Plasma clearance of sulfobromophthalein and its interaction with hepatic binding proteins in normal and analbuminemic rats: Is plasma albumin essential for vectorial transport of organic anions in the liver?

(hepatic anion transport/biliary secretion/albumin receptor)

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ABSTRACT **To investigate a possible function of plasma albumin in the vectorial transport of organic anions by the liver, the plasma disappearance of sulfobromophthalein (BSP) and its interaction with plasma and liver cytosolic proteins were studied in normal rats and mutant Nagase analbuminemic rats (NAR). After intravenous administration of BSP, plasma BSP decreased rapidly in both NAR and control animals: plasma clearance values of BSP in NAR and controls were 12.45 and 7.40 ml/min per kg, respectively. Gel exclusion Sephadex G-100 chromatography of BSP with control rat serum revealed a protein peak in the void volume and another in the albumin fraction. BSP chromatographed exclusively with the albumin fraction; binding of BSP to plasma albumin occurred stoichiometrically. Similar studies with NAR serum revealed a single protein peak, in the void volume; a small amount of BSP chromatographed with this protein peak. The amount of BSP that chromatographed with NAR serum protein(s) was 8% of that with control rat serum albumin. Sephadex G-100 chromatography of BSP with control rat liver cytosol revealed four peaks of protein-bound BSP in fractions corresponding to the void volume (fraction X), albumin, glutathione S-transferases (fraction Y, M_r 45,000), and fraction Z (M_r 12,000); fraction Y was the major component of BSP binding. Gel chromatography of NAR liver cytosol with BSP revealed three BSP peaks, fractions X, Y, and Z; fraction X was the major component of BSP binding. Total BSP binding by 30 mg of hepatic cytosolic proteins was 4.5 nmol for controls and 10.4 nmol for NAR. Isoelectric focusing of liver cytosol revealed no quantitative or qualitative differences in glutathione S-transferase isozymes between control and mutant animals. Intravenously administered BSP (5 μmol/kg) rapidly appeared in bile as the free form and the glutathione conjugate in normal rats and NAR; 41% and 57% of injected BSP was excreted within 60 min in NAR and control rat bile, respectively. These results indicate that binding of BSP to plasma albumin is not indispensable to transhepatocyte transport of BSP *in vivo*.**

Many hydrophobic anions, such as bilirubin, bile acids, and xenobiotics, are efficiently removed from plasma during passage through the liver. Their uptake into hepatocytes involves carrier-mediated mechanisms (1). Since most of these ligands tightly bind to plasma albumin, their transport has been believed to follow dissociation from albumin with interaction of free ligands with "translocases" in hepatocyte sinusoidal plasma membrane (1, 2). However, it remains obscure whether or not binding by plasma albumin is essential for vectorial transport of hepatophilic organic anions.

Recently, mutant Nagase analbuminemic rats (NAR) were described (3). Despite the absence of plasma albumin, the mutants show no remarkable sign of pathology except for hyperlipidemia and mild anemia (3, 4). Total serum protein concentration of NAR is similar to that of control Sprague–Dawley rats due to an increased globulin fraction (3). There is impaired processing of albumin mRNA precursor (5, 6).

To elucidate the vectorial transport mechanism of organic anions by the liver, the *in vivo* fate of intravenously injected sulfobromophthalein (BSP) and interaction of BSP and its glutathione (GSH) conjugate with plasma and liver cytosolic proteins were studied in normal rats and NAR.

MATERIALS AND METHODS

Materials. Bovine serum albumin (essentially fatty acid free), GSH, and BSP were obtained from Sigma. Heparin was purchased from Nakarai Chemicals (Kyoto, Japan). All reagents used were of analytical grade. The GSH conjugate of BSP (GS-BSP) was synthesized from GSH and BSP (7) and was free from BSP as determined by thin-layer chromatography in propan-1-ol/water (7:3, vol/vol).

Plasma Clearance of BSP. Male Sprague–Dawley and NAR mutant animals, 180–220 g, were fasted for 16 hr prior to operation. *In vivo* experiments were performed between 9:00 a.m. and 12:00 noon while the animals were under pentobarbital anesthesia (50 mg/kg of body weight). Five minutes before experiments, animals were heparinized intravenously (2,500 units/kg). BSP was dissolved in 0.15 M NaCl and injected into the right femoral vein (5 μmol/kg) over a period of 5 sec. At timed intervals, 0.1-ml blood samples were collected from the left femoral vein and centrifuged at 15,000 rpm for 3 min in an Eppendorf 5412 centrifuge as described (8). Plasma BSP concentrations were determined spectrophotometrically at 580 nm in 0.1 M NaOH (7).

Biliary Excretion of BSP and GS-BSP. Biliary excretion of BSP was determined in pentobarbital-anesthetized rats that had common bile duct cannulation for 60 min after intravenous administration of BSP. BSP was determined in bile samples during 12 consecutive 5-min collection periods. Thin-layer chromatographic analysis of bile from control and NAR animals contained BSP and GS-BSP; the relative amounts of free and conjugated BSP were similar in the two groups of bile samples. During *in vivo* experiments, body temperature of anesthetized animals was kept constant by using a tungsten lamp.

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Abbreviations: NAR, Nagase analbuminemic rat(s); BSP, sulfobromophthalein; GSH, glutathione; GS-BSP, glutathione S-sulfobromophthalein.

Liver Cytosolic Fractions. Animals were killed by bleeding from the femoral artery, and the liver was perfused *in situ* with 20 ml of ice-cold 0.15 M NaCl. The liver was excised, minced, and homogenized in 5 vol (wt/vol) of 0.25 M sucrose/10 mM Hepes–Tris buffer, pH 7.4/0.2 mM $CaCl_2$ with 12 strokes in a loose-fitting Dounce homogenizer at 0°C. After filtration through cheesecloth, ethylenediaminetetraacetic acid (EDTA) was added to give a final concentration of 1 mM. The homogenate was centrifuged at 43,000 × *g* for 30 min at 4°C. The supernatant fraction was centrifuged again, at 105,000 × *g* for 2 hr. The supernatant fraction was carefully collected to avoid the lipid layer floating on top of the centrifuge tubes. The cytosolic fraction was stored at −70°C until used.

BSP Binding Study. Binding of BSP to serum and liver cytosolic proteins was analyzed by gel filtration of samples on a Sephadex G-100 column (3 × 30 cm) (9). The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.4, and chromatography was performed with the same buffer solutions at 20 ml/hr. Fractions (2 ml) were collected and protein and BSP concentrations were determined.

Isoelectric Focusing of GSH *S*-Transferases. Ammonium sulfate was added to liver cytosolic fractions to give a final concentration of 70% saturation, and the mixtures were centrifuged at 10,000 × *g* for 20 min at 4°C. The precipitates were dissolved in water and dialyzed against water for 16 hr at 4°C. Dialyzed samples were centrifuged at 10,000 × *g* for 20 min to eliminate insoluble materials and used for isoelectric focusing (pH range of 3.0–10.0) (10).

Assay for Enzyme Activity. GSH *S*-transferase activity was measured at 25°C by using 2,4-dinitrochlorobenzene and GSH (11). Protein concentrations were determined by the method of Lowry *et al.* (12) with bovine serum albumin as the standard.

RESULTS

Plasma Clearance of BSP. As shown in Fig. 1, the plasma level of BSP decreased rapidly in both NAR and control animals. Thirty seconds after administration of BSP, plasma BSP concentration reached 0.1 and 0.18 mM in NAR and control animals, respectively. Thereafter, plasma BSP levels decreased at similar rates in the two animal groups. From semilogarithmic plots of BSP disappearance, apparent plasma half-lives of 2.4 and 2.3 min were calculated for NAR and control animals, respectively (Fig. 1*B*). By measuring the area under the BSP disappearance curve and using the trapezoidal rule (13), values of 12.45 ml/min·kg in NAR and 7.40 ml/min·kg in control animals were calculated for the clearance of BSP at a dose of 1 μmol/200 g of body weight. Thus, plasma BSP clearance was significantly greater in NAR than in control animals.

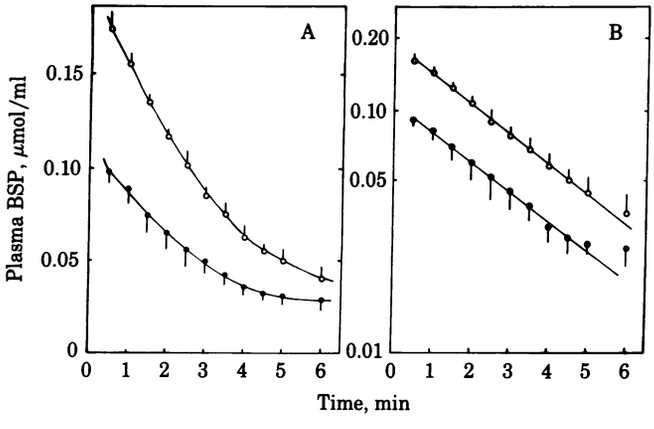

FIG. 1. Plasma clearance of BSP. (*A*) After intravenous administration of BSP (5 μmol/kg) to normal rats (○) and NAR (●), blood samples (0.1 ml) were obtained at indicated times and the plasma BSP level was determined. Each point represents mean value ± SD derived from triplicate experiments. (*B*) Semilogarithmic plots of *A*.

Biliary Excretion of Injected BSP. Intravenously administered BSP accumulates preferentially in liver and then is excreted into bile. As shown in Fig. 2, intravenously injected BSP rapidly appeared in bile in control animals. The biliary BSP level was maximal within 10 min of administration and decreased thereafter; about 58% of the injected dose was recovered in control rat bile within 60 min of administration. NAR revealed similar excretory profiles of intravenously administered BSP; 41% of the injected dose was recovered in bile within 60 min of administration. Despite lack of plasma albumin, overall transport of BSP from plasma into bile was not affected significantly in NAR. Sixty minutes after administration, no significant BSP was found to remain both in plasma and liver cytosol of control rats and NAR, indicating that 42–59% of the injected dose remained in extrahepatic tissues.

BSP Binding to Plasma Proteins. To elucidate the mechanism of the rapid decrease in initial plasma BSP level in NAR, BSP binding to serum proteins was studied by using gel exclusion chromatography on Sephadex G-100. Control rat serum revealed two protein peaks; one was in the void volume fraction and the other was in an inner volume fraction corresponding to albumin (Fig. 3). When 0.5 ml of serum was used, about 250 nmol of BSP chromatographed with the second protein peak. In contrast, gel chromatography of NAR serum with BSP revealed a single protein peak, in the void volume; a small amount of BSP (20 nmol) chromatographed with this protein peak under conditions identical to those for control serum. Although an equal amount of serum (0.5 ml) was used, the 280-nm absorbance for the void volume fraction was 2-fold greater in NAR than in control rat serum (Fig. 3). This is consistent with previous observations that the total serum protein concentration in NAR is similar to that in control rats due to increase in globulins (3).

Similar binding experiments were also performed by varying

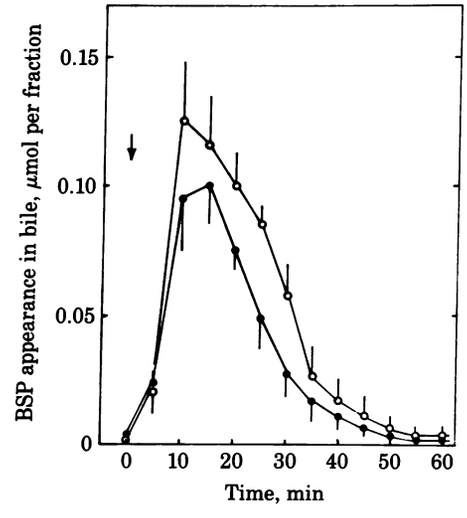

FIG. 2. Biliary excretion of BSP. After intravenous administration of BSP (5 μmol/kg), its biliary excretion was determined in normal rats (○) and NAR (●). Biliary BSP was measured for samples of 12 consecutive 60-min collection periods. Bile duct cannulation was performed while the animals were under pentobarbital anesthesia 60 min prior to experiments. Bile flow remained constant during the experiments (1.5–2 μl/min per g of liver). Values on the ordinate show total amounts of BSP (free form plus + GSH conjugate) per fraction. Each point represents mean ± SD derived from triplicate determinations.

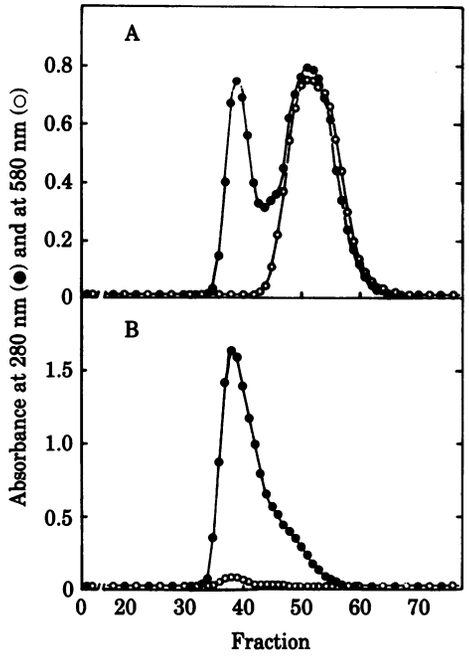

FIG. 3. Binding of BSP to serum proteins. After incubation of 0.25 μmol of BSP at 25°C for 5 min with 0.5 ml of the control rat (*A*) or NAR (*B*) serum, the samples were subjected to gel chromatography on Sephadex G-100 (3 × 30 cm) at 4°C. Fractions (2 ml) were collected and protein (●) and BSP (○) were determined. After each experiment, the Sephadex G-100 column was washed with an amount of bovine serum albumin sufficient to eliminate BSP nonspecifically bound to the matrix and then the column was equilibrated with the elution buffer.

BSP concentrations from 0.025 to 2.5 μmol at a fixed amount of serum (0.5 ml). However, no significant change in gel chromatographic profiles of the two serum samples was observed except that BSP bound to control serum albumin increased with the increase in BSP concentration. BSP binding to albumin reached saturation at 0.5 μmol of BSP per ml of control serum. Above this BSP concentrations, the excess ligand remained bound to the Sephadex G-100 column; this BSP was eluted quantitatively by washing the column with a solution containing an excess of bovine serum albumin. The results indicate that a large proportion of injected BSP was rapidly eliminated from plasma by tissue(s) as a result of low BSP-binding activity of NAR serum protein(s).

BSP Binding to Liver Cytosolic Proteins. Gel exclusion chromatography of normal rat liver cytosol with BSP revealed that three peaks of protein bound BSP: fractions X, Y, and Z. Fraction Y was the major component of BSP binding. Other BSP-binding fractions were observed at positions corresponding to GSH S-transferases or ligandins (14). An additional BSP peak was seen between X and Y fractions (Fig. 4*A*), which is the position for albumin (15). Gel chromatography of NAR liver cytosol with BSP also revealed three peaks of protein-bound BSP (Fig. 4*B*); fraction X was the major component of BSP binding. BSP in fraction X was about 2- to 3-fold greater than in fraction Y of NAR liver cytosol.

BSP is conjugated *in vivo* with GSH; the reaction is catalyzed by hepatic GSH S-transferases (16). Since GS-BSP is more hydrophilic and less toxic than free BSP (17), the two dyes may interact differently with liver cytosolic binding proteins. To test this possibility, GS-BSP was chromatographed with control or NAR liver cytosol on Sephadex G-100 (Fig. 5). GS-BSP chromatographed with two protein peaks, which corresponded to fractions X and Y of control liver cytosol (Fig. 5*A*). GS-BSP associated with fraction X was 14% of that associated with fraction

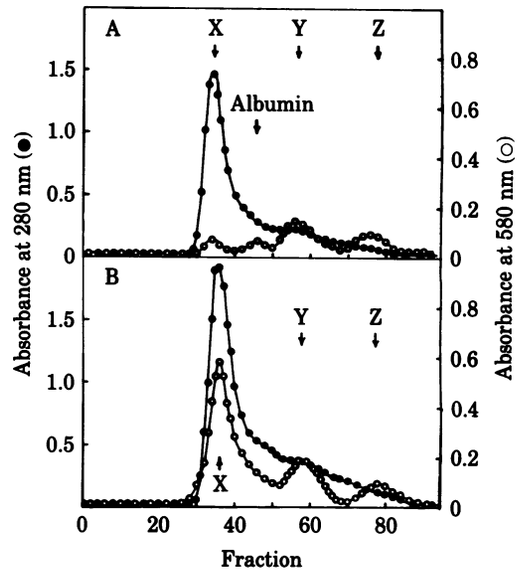

FIG. 4. Binding of BSP to hepatic cytosolic proteins. After incubation of 0.25 μmol of BSP with 2 ml of cytosolic fractions (30 mg of protein) obtained from normal rat (*A*) and NAR (*B*) liver at 25°C for 5 min, the samples were subjected to gel exclusion chromatography on Sephadex G-100 at 4°C. Fractions (2 ml) were collected and protein (●) and BSP (○) were determined as in Fig. 3.

Y; GS-BSP was not detected in fraction Z. Gel filtration of NAR liver cytosol also revealed two peaks of protein-bound GS-BSP in fractions X and Y (Fig. 5*B*). The amount of GS-BSP chromatographed with fraction X was 1.2-fold greater than that with fraction Y. GS-BSP recovered in fraction Y was similar in the two cytosolic samples. Thus, a striking difference in both BSP and GS-BSP binding between the liver cytosols from control rats and NAR is that fraction X in the latter showed a considerably higher capacity for binding organic anions.

Isoelectric Focusing of GSH S-Transferase Isozymes. GSH S-transferase (fraction Y) is responsible for binding of organic

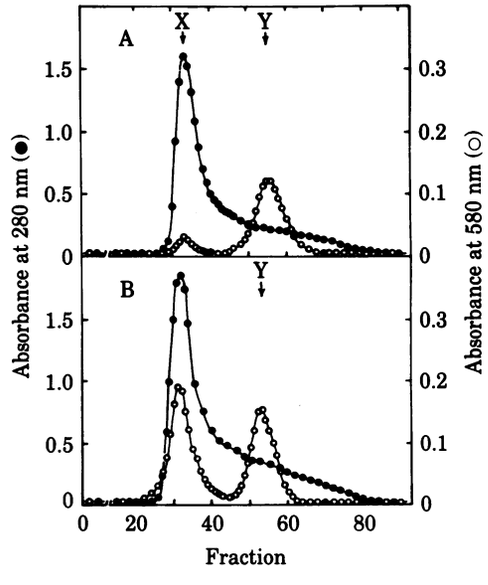

FIG. 5. Binding of GS-BSP to hepatic cytosolic proteins. After incubation of 0.25 μmol of GS-BSP with 2 ml of cytosolic fractions (30 mg of protein) obtained from normal rat (*A*) and NAR (*B*) liver at 25°C for 5 min, the samples were subjected to gel filtration chromatography on Sephadex G-100 (3 × 28 cm) at 4°C. Fractions (2 ml) were collected and protein (●) and GS-BSP (○) were determined as in Fig. 3.

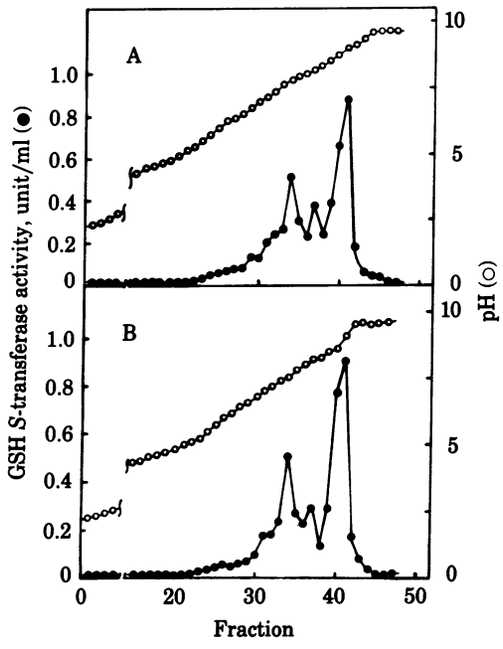

FIG. 6. Isoelectric focusing of GSH *S*-transferase isozymes. The ammonium sulfate fractions of liver cytosol obtained from the control rats (*A*) or NAR (*B*) were subjected to isoelectric focusing at 4°C in the pH range 3.0–10.0 as described in ref. 10. Each sample contained 8 units of GSH *S*-transferase activity as determined with 1 mM GSH and 2,4-dinitrochlorobenzene. After isoelectric focusing, 1.5-ml fractions were collected and enzyme activity (●) and pH (○) were determined.

anions, and it consists of several isozymes, AA, A, B, C, D, and E (11). To test the possibility that physicochemical properties or isozymic profiles of GSH *S*-transferases in NAR liver cytosol differ from those in control rat liver cytosol, we compared transferase isozymes in the two cytosolic fractions. GSH *S*-transferases in the two cytosol fractions had similar activities as determined with 2,4-dinitrochlorobenzene and GSH; about 12–15 units/g of liver (wet weight) was obtained for transferase activity in control or NAR liver. The isozymic profiles of transferases in control and NAR liver cytosol were also similar (Fig. 6). Thus there seems to be no significant quantitative and qualitative differences in transferase isozymes between the control and mutant NAR liver.

DISCUSSION

The present work demonstrated that intravenously administered BSP was taken up by the liver and rapidly excreted into bile in NAR and control rats. The rate of plasma clearance of the injected BSP was significantly greater in NAR than in control animals. Preliminary experiments in which BSP bound to albumin (1:1) was injected into NAR (5 μmol/kg of body weight) revealed that plasma BSP clearance was identical to that in control animals that were injected with free BSP. Thus it seems likely that the presence of an apparently larger volume for BSP distribution in NAR than in control animals results from low BSP binding activity of NAR serum protein(s) with the consequent rapid binding of free BSP to various tissues.

Gel chromatographic analysis revealed that the capacity of BSP binding increased markedly in NAR liver cytosol as compared with that in control. This high capacity is due to an increase in the binding activity of fraction X (Figs. 4 and 5) of NAR liver cytosol. The protein molecule(s) in fraction X responsible for this binding are not known at present. Despite a marked increase in BSP binding activity of NAR liver cytosol, the rate of translocation from plasma into hepatocytes remained unchanged as judged from the plasma half-life and biliary secretion of BSP. These data suggest that intracellular binding of BSP may not affect the rate of hepatic transport across the sinusoidal plasma membranes. This observation is consistent with the proposal that intracellular binding of solutes to cytoplasmic proteins may influence steady-state levels without affecting ligand transport process (18).

In the circulation, BSP and other organic anions, including bilirubin, are avidly bound to albumin, from which they are extracted by the liver (1, 2, 19). The role of albumin in hepatic uptake of these organic anions is controversial. Several studies with these hepatophilic anions suggest that it is the small unbound fraction that interacts with hepatocytes and is removed readily from the circulation. On the basis of kinetic analysis of taurocholate uptake by isolated perfused liver, Forker and Luxon (20) postulated that albumin interacts with the hepatocyte surface, accelerates the release of taurocholate, and facilitates its subsequent uptake. Thus the presence of an albumin receptor in hepatic sinusoidal plasma membranes has been suggested (21). However, Stremmel *et al.* (22) observed that binding of ^{125}I-labeled albumin (native and defatted samples) to hepatic plasma membranes was no greater than to erythrocyte ghosts, was not inhibited by excess unlabeled albumin, and was not decreased by heat denaturation of the sinusoidal membranes. Stollman *et al.* (23) showed that hepatic uptake of bilirubin does not require binding to albumin; uptake of radioactive bilirubin injected bound to ligandin, an intracellular high-affinity binding protein, was as rapid as that injected bound to albumin. These observations are consistent with the present findings that transhepatocyte transport of BSP occurs in both NAR and control animals. Apart from the possible presence of a putative albumin receptor, it is apparent that plasma albumin is not indispensable for hepatic translocation of BSP across sinusoidal and canalicular membranes.

The biliary recovery of injected BSP was somewhat greater in control animals than in NAR (Fig. 2). As compared with administration of free BSP, injection of BSP bound to albumin (1:1) into NAR (5 μmol/kg of body weight) resulted in higher recovery; about 70% of the injected dose was excreted in bile within 60 min. Thus the role of albumin in the vectorial transport of hepatophilic organic anions may be to retain these solutes in plasma in their bound form, from which the high-affinity transport systems in the sinusoidal plasma membrane can effectively extract them from the circulation. This albumin binding may also participate in protecting extrahepatic tissues from toxic effects of these amphipathic molecules caused by their nonspecific binding. That animals obtained from crossing NAR and jaundiced Gunn rats have no plasma albumin and die with kernicterus within 3 weeks after birth (24) is consistent with the notion described above.

It should be noted that the present experiments *in vivo* dealt with the overall clearance of BSP from the systemic circulation. The ligand transport has been studied in detail in isolated perfused liver (2, 23) and hepatic plasma membrane vesicles obtained from normal rat liver (25, 26). Similar studies of BSP transport using NAR should shed further light on the mechanism of hepatic clearance.

Hepatophilic organic acids (such as bilirubin, bile acids, fatty acids, indocyanine green, and biligraphic agents) and nephrophilic anions (such as *p*-aminohippuric acid, phenolsulfophthalein, and urographic agents) bind to plasma albumin and are selectively translocated from plasma into bile or urine via specific vectorial transport systems in liver and kidney (27). Thus this analbuminemic mutant animal will provide important information about transepithelial transport of these ligands *in vivo*

and serves as a model for understanding the pathophysiology of human analbuminemic or hypoalbuminemic diseases (28–30).

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