Rapid Publication

Irreversible Binding of Conjugated Bilirubin to Albumin in Cholestatic Rats

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Abstract. A diazo-positive fraction of serum bilirubin that is irreversibly bound to albumin has been shown to accumulate in serum of patients with cholestasis. In the present study, a cholestatic animal model was used to determine the chemical nature of the bilirubin species involved in its formation. The data indicate that conjugated bilirubin is the precursor of "albumin-bound bilirubin" and that the presence or absence of light does not affect its formation. An albumin-bound bilirubin-complex indistinguishable from the complex detected in cholestatic sera from patients or in bile duct-ligated Sprague-Dawley rats can be formed in vitro in sera enriched in conjugated bilirubin at 37°C, pH 7.4.

Introduction

The serum levels of unconjugated bilirubin as well as bilirubin monoglucuronide and diglucuronide are increased in patients with cholestasis. In normal sera, these tetrapyrroles exist bound to albumin through reversible ionic bonds (1, 2). Recently, a direct-reacting, diazo-positive bilirubin compound that is irreversibly bound to albumin was detected and quantified in sera of patients with cholestatic diseases (3, 4). A similar compound was previously described by both Nosslin (5) and Kuenzle et al. (6) but their observations were overlooked until recently.

This bilirubin species, albumin-bound bilirubin, is detected as a significant fraction of total serum bilirubin in jaundiced patients with a variety of hepatobiliary diseases, but is not detected in healthy volunteers, in neonates with physiological jaundice, or in patients with hemolysis or Gilbert's disease (4). Our findings suggested that conjugates of bilirubin may be the source of this compound. In addition, during recovery from jaundice, albumin-bound bilirubin persists and eventually becomes the predominant fraction of total bilirubin in serum. This phenomenon probably occurs because of the tight binding to albumin so that the turnover of albumin then determines the compound's half-life in serum; this could explain the well-known delay in resolution of jaundice in some patients who have recovered normal hepatobiliary function (4).

In the present study, an animal model for albumin-bound bilirubin was used to investigate the chemical nature of the bilirubin species and the site of formation of albumin-bound bilirubin. Bile duct-ligated Sprague-Dawley rats and homozygous Gunn rats were chosen for these experiments. In the Sprague-Dawley rats, the level of both unconjugated and conjugated bilirubin would increase in serum, while in the Gunn rats, only unconjugated bilirubin would increase. Additionally, the effect of an infusion of unconjugated bilirubin and phototherapy on the formation of albumin-bound bilirubin was compared in the two groups of animals.

Methods

Chemicals. Bilirubin was obtained from Sigma Chemical Co. (St. Louis, MO); saccharo-1-4-lactone, from Calbiochem-Behring (La Jolla, CA); 25% human serum albumin, from Cutter Laboratories (Berkeley, CA); Amberlite CG-400 anion-exchange resin, from Mallinckrodt Chemical Works (St. Louis, MO) and penicillin/streptomycin (5,000 U/5,000 μg/ml), from Grand Island Biologicals (Grand Island, NY). Normal human serum was obtained from fasting, healthy laboratory employees.

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Animals. Male Sprague-Dawley rats (200–250 g, Charles River Laboratories, Wilmington, MA) and male, homozygous Gunn rats (200–300 g, Blue-Spruce Farms, Albany, NY) were housed in temperature- and humidity controlled rooms and had free access to water and Purina rat chow (Ralston Purina Co., Chicago, IL). Animals were not starved before surgery.

Surgical procedures. Under ether/ketamine anesthesia, the bile duct was cut between double ligatures above the entrance of the pancreatic ducts. After surgery, the animals were housed in individual cages. In the initial postoperative phase (1–2 d), the animals lost up to 10% of their body weight before resuming weight gain. Blood samples for determination of albumin-bound bilirubin and total bilirubin were obtained from the tail vein at 24–48-h intervals. The serum was separated and analyzed immediately or frozen at −20°C and analyzed within 24 h.

Preparation and infusion of conjugated and unconjugated bilirubin

Unconjugated bilirubin. 25 mg (42.7 μmol) of bilirubin was dissolved in 0.20 M NaOH, added to enough 25% human serum albumin to give a final concentration of 5% human serum albumin and then neutralized to pH 7.5 with dilute HCl to yield an isotonic solution containing 1.25 mg/ml of bilirubin. On high pressure liquid chromatography (7), extracts of this material chromatographed as unconjugated bilirubin IX-α. Infusions of this material were made into the jugular vein of rats with a size 23 butterfly needle at the rate of 2–3 ml/h (4–6 μmol of bilirubin/h) using a Harvard infusion pump (Harvard Apparatus, Dover, MA).

Conjugated bilirubin. Bile, containing high concentrations of conjugated bilirubin, was collected on ice in the dark via a polyethylene bile duct-cannula (PE-50 tubing, Clay-Adams, Parsippany, NJ) from Sprague-Dawley rats that were infused with 4–6 μmol of bilirubin. Saccharo-1-4-lactone (1 mM) was added and samples frozen immediately and used within 24–48 h. These samples contained 100–150 mg/100 ml of bilirubin conjugates. High pressure liquid chromatographic analysis of these enriched bile samples (7) revealed no significant unconjugated bilirubin and these samples contained 65% diglucuronide and 35% monoglucuronide. These bile samples were used as a source for bilirubin conjugates and in experiments requiring infusion of conjugated bilirubin, 2–3 ml of enriched bile, containing 3–6 μmol of bilirubin were infused at 2.5 ml/h.

Purified conjugates of bilirubin were prepared from bile as outlined by Berk et al. (8) and used in some in vitro incubations. These samples contained primarily bilirubin monoglucuronide and diglucuronide by thin-layer chromatography (9).

Phototherapy. Gunn rats were shaved and exposed to a flux of 20 $\mu W/cm^2$ per nm, provided by banked Daylite fluorescent lamps (Sylvania Electronic Products & Services, Williamsport, PA) kept at 24 in. from skin surface for two 48-h periods separated by a 24-h recovery period.

In vitro incubation of bilirubin samples with human serum. Samples of unconjugated bilirubin (19–36 mg/100 ml), bile enriched with bilirubin conjugates and purified conjugates (28–60 mg/100 ml) were incubated for 24 h with normal fasting human serum at 37°C, pH 7.4 under nitrogen in sealed tubes containing penicillin/streptomycin (100 U/100 μg per ml). This bilirubin/serum mixture was also analyzed immediately after mixing or after being maintained at 25°C.

Measurement of albumin-bound bilirubin. Albumin-bound bilirubin was measured chemically in samples by a method developed by Seligson, H., D. Seligson, and T.-W. Wu (submitted for publication). Briefly, cholestatic serum samples (150 μl), containing bilirubin, its conjugates, and albumin-bound bilirubin, were diazotized according to the Doumas et al. (10) modification of the Jendrassik-Grof diazo reaction. Blanks were treated identically except for the omission of $NaNO_2$. After addition of the alkaline tartarate reagent, the diazotized serum was added to 50 mg of prepared (chloride free) anion-exchange resin, shaken for 25 min in the dark, and then filtered twice to remove azoderivatives of the bilirubin fractions that were not bound covalently to albumin. Azoderivatives of albumin-bound bilirubin were not retained by this resin. The effluent from the ion-exchange resin was measured spectrophotometrically at 660 nm and albumin-bound bilirubin was quantified after subtracting the absorbance of the blank. Validation of the photometric method was obtained by comparison of values of albumin-bound bilirubin obtained by this method on 54 jaundiced serum samples, with values obtained by the high pressure liquid chromatographic method of Lauff et al. (11) on the same samples. The data from the photometric method of Seligson, H., D. Seligson, and T.-W. Wu (submitted for publication) were in good agreement with the specific method of Lauff et al. (11), yielding a correlation coefficient of 0.965. Additional proof of the method was provided by measurements made with purified albumin-bound bilirubin, isolated from pooled jaundiced human sera as described previously by Lauff et al. (11).

Analysis of results. All values are arithmetic means. Statistical analyses were performed by the *t* test.

Results

Negligible amounts of albumin-bound bilirubin were detected in conjugated bilirubin standards (total bilirubin, 21–65 mg/100 ml; albumin-bound bilirubin, 0.5–1.1%) or in conjugated bilirubin derived from rat bile (total bilirubin, 31–208 mg/100 ml; albumin-bound bilirubin 0–0.3%). Similarly, as shown in Table I, negligible amounts of albumin-bound bilirubin were detected in reaction mixtures containing unconjugated bilirubin (513±87 μmol/liter) in human serum or conjugated bilirubin (584±102 μmol/liter) in sera which were assayed immediately (0.11±0.06%, $n = 5$; 1.03±1%, $n = 7$, respectively, expressed as percentage of total bilirubin).

Formation of albumin-bound bilirubin

In vivo formation in cholestatic rats. Total serum bilirubin rose to similar peak levels of 16.0±3 mg/100 ml and 16.8±3.2 mg/100 ml within 24–72 h after bile duct-ligation in Sprague-Dawley and Gunn rats, respectively (Fig. 1 *A*). The initial serum bilirubin levels were significantly higher in Gunn rats and after ligation of bile ducts, the serum values increased more rapidly than in Sprague-Dawley rats. However, in both experimental groups, this initial rise was followed by a slow decline in total serum bilirubin. This phenomenon has been described previously in the bile duct-ligated rat model (12) and presumably is a result of an enhanced efficiency of nonhepatic routes for bilirubin excretion. In Sprague-Dawley rats, albumin-bound bilirubin was detected within 24 h of bile duct-ligation (0.73±0.27 mg/100 ml; 12.5±4.6 μmol/liter of bilirubin) and this level increased slowly during the experimental period to account for 40–45% of total serum bilirubin on the 10th d (3.8±0.31 mg/100 ml). In contrast, albumin-bound bilirubin was not detected in sig-

Table I. Formation of Albumin-bound Bilirubin In Vitro

Substrate	Conditions		Products	
	Time	Temperature	Total bilirubin	Albumin-bound bilirubin
	h	*°C*	*mg/100 ml*	*% of total bilirubin*
Conjugated bilirubin (n = 9)	24	37	38.6±12.2	4.04±1.5
Conjugated bilirubin (n = 9)	24	25	30.2±1.8	2.70±0.6*
Unconjugated bilirubin (n = 7)	24	37	30.1±5.5	0.80±0.5‡
Conjugated bilirubin (n = 7)	0	25	33.6±6.0	1.03±1.0
Unconjugated bilirubin (n = 5)	0	25	30.0±5.1	0.11±0.06

Indicated values are means±SD. Comparison was made between albumin-bound bilirubin formed on incubation of conjugated bilirubin with albumin at 37°C and (*a*) incubation at 25°C (*$P < 0.02$) and (*b*) incubation of unconjugated bilirubin with albumin at 37°C (‡$P < 0.001$).

nificant amounts in sera of bile duct-ligated Gunn rats at any point during the experimental period.

Total serum bilirubin was significantly increased in both bile duct-ligated Sprague-Dawley and Gunn rats after infusion of 2 μmol/100 g body wt i.v. of bilirubin over 60–90 min (Fig. 1 *B*). In Sprague-Dawley rats, this was associated with a significant enhancement in the amount of albumin-bound bilirubin detected 24 h after infusion (2.84±0.7 mg/100 ml, which was significantly greater than that found in Sprague-Dawley rats after bile duct-ligation alone, $P < 0.001$). Again, virtually no albumin-bound bilirubin was detected in sera of the bile duct-ligated Gunn rats infused with bilirubin and treated in the same manner.

When bile samples enriched in bilirubin conjugates (containing 1 mM saccharo-1-4-lactone) were infused into Sprague-Dawley rats, significantly increased amounts of albumin-bound bilirubin were detected at 24 h (2.8±0.4 mg/100 ml) (Fig. 2). Albumin-bound bilirubin was also readily detected in sera of bile duct-ligated Gunn rats (1.3±0.5 mg/100 ml at 24 h). This experiment represented the only experimental condition in which albumin-bound bilirubin was detected in cholestatic Gunn rats.

To study the effect of light on albumin-bound bilirubin formation, Sprague-Dawley rats were kept in complete dark after bile duct-ligation (Fig. 3, left) and compared with rats kept in a normal, 12-h alternating light-dark cycle after surgery. There was no significant difference, either in total bilirubin or

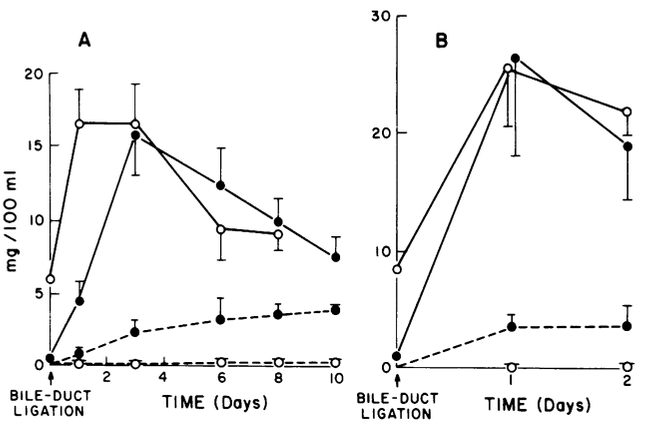

Figure 1. (*A*) Total serum bilirubin (solid lines) and albumin-bound bilirubin (dashed lines) in Spraque-Dawley (●) (n = 14) and homozygous Gunn rats (○) (n = 7) following bile duct-ligation. Range bars indicate means±SD. (*B*) Total serum bilirubin and albumin-bound bilirubin in bile-duct ligated Sprague-Dawley (●) (n = 7) and Gunn (○) (n = 3) rats after intravenous infusion of 2 μM/100 g body wt of unconjugated bilirubin. Range bars indicate means±SD.

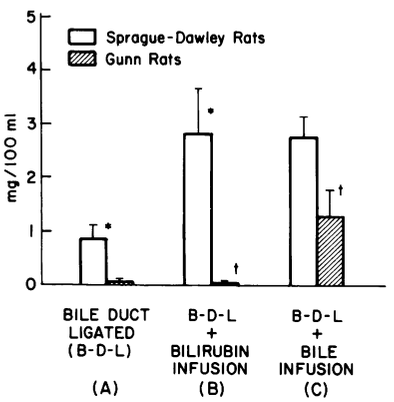

Figure 2. Albumin-bound bilirubin (mg/100 ml) in serum of Sprague-Dawley and Gunn rats 24 h after (*A*) bile duct-ligation alone; (*B*) bile duct-ligation plus infusion of 2 μM/100 g body wt of unconjugated bilirubin showing significantly enhanced formation of albumin-bound bilirubin in Sprague-Dawley rats (*$P < 0.01$); and (*C*) bile duct-ligation plus infusion of bile enriched in bilirubin conjugates, showing detectable and significantly higher albumin-bound bilirubin formation in Gunn rats (†$P < 0.001$).

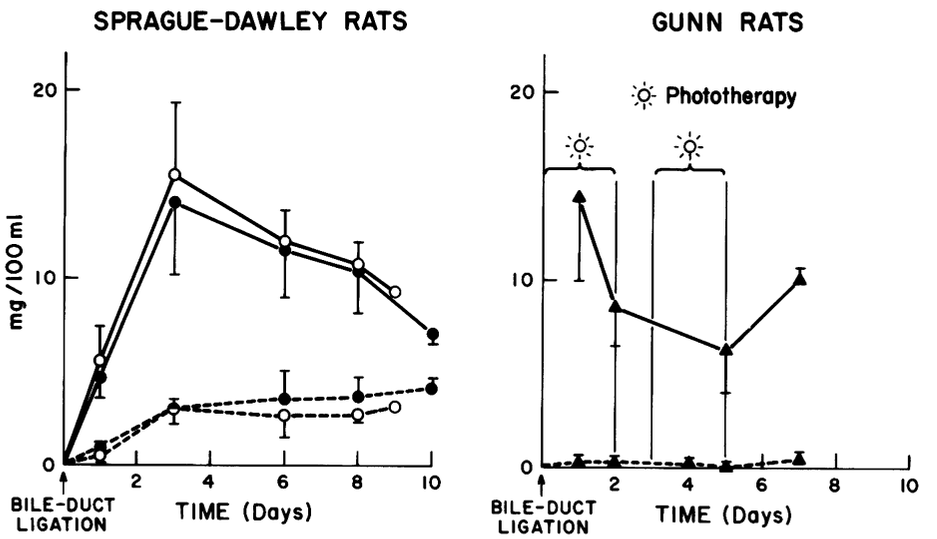

Figure 3. Effect of exposure to light on albumin-bound bilirubin formation. (*Left*) Comparison of total bilirubin (solid lines) and albumin-bound bilirubin (dashed lines) in serum from cholestatic Sprague-Dawley rats kept in complete dark (n = 4) (●) with rats exposed to a normal 12-h light-dark cycle (n = 10) (○). (*Right*) Albumin-bound bilirubin levels after exposure of bile duct-ligated Gunn rats to phototherapy (n = 5). Total serum bilirubin falls with phototherapy and increases after cessation.

in albumin-bound bilirubin levels in the two groups. In Gunn rats (Fig. 3, right), total bilirubin rose after bile duct-ligation and declined with phototherapy. However, there was no significant albumin-bound bilirubin formation.

In vitro formation. In vitro incubation of conjugated but not unconjugated bilirubin at 37°C, pH 7.4 for 24 h resulted in the tight binding of 3–6% of bilirubin to albumin (Table I). At room temperature, this in vitro formation of albumin-bound-bilirubin–like material was significantly decreased (2.7±0.6% of total bilirubin, $P < 0.02$).

Discussion

The present study confirms, in an animal model, our earlier observation in patients that albumin-bound bilirubin is detected in serum in the presence of conjugated but not unconjugated hyperbilirubinemia (4). The ready formation of albumin-bound bilirubin in cholestatic Sprague-Dawley rats (with an intact bilirubin conjugating mechanism) and Gunn rats infused with conjugated bilirubin indicates that bilirubin conjugates are the direct precursors of albumin-bound bilirubin formation.

Covalent linkage of light-irradiated bilirubin to albumin has been reported to occur both in vivo and in vitro (13). However, our results indicate that exposure to phototherapy does not result in formation of detectable amounts of albumin-bound bilirubin. In this study no attempt was made to measure bilirubin photoisomers which accumulate in serum of Gunn rats after phototherapy (14). Their formation and excretion, into urine and the intestine, may be partly responsible for the fall in total serum bilirubin seen after phototherapy. Detection of albumin-bound-bilirubin–like substance(s) on incubating conjugated bilirubin with albumin indicates that a tightly bound bilirubin-albumin complex can be formed in vitro by a temperature-dependent, nonenzymatic mechanism. This compound is indistinguishable from albumin-bound bilirubin by the ion-exchange resin-filtration method used in this study that selectively measures azoderivatives of bilirubin coupled irreversibly to albumin. Preliminary evidence obtained in this laboratory from sodium dodecyl sulfate-polyacrylamide electrophoresis of albumin-bound ^{3}H-bilirubin samples further supports the similarity of the compounds formed in vivo and in vitro. ^{3}H-bilirubin counts are predominantly associated with the albumin band in both samples of albumin-bound bilirubin obtained from in vivo (bile duct-ligated rat) sources and those obtained on incubation of ^{3}H-conjugated bilirubin with normal serum in vitro.

Further studies are required to determine if the in vivo formation of albumin-bound bilirubin occurs in the liver or in peripheral circulation; if the albumin-bound bilirubin compound formed in vitro is the same as that formed in vivo; and to determine the overall pathophysiological significance of this unique, tight binding of a conjugated bilirubin species to albumin.

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