Non-Resolving Jaundice: Bilirubin Covalently Attached to Serum Albumin Circulates with the Same Metabolic Half-Life as Albumin

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In hepatobiliary disease and biliary obstruction, bilirubin often becomes covalently bound to albumin circulating in serum, producing a nondissociable complex. To determine how long this complexed bilirubin remains in the circulation, we compared the metabolic clearance of bilirubin–albumin complexes with the clearances of free bilirubin and unmodified albumin. Radiolabeled bilirubin, albumin, and covalent bilirubin–albumin were injected into the circulation of Sprague-Dawley rats and serial samples of plasma were analyzed for the injected compounds. The half-life of bilirubin was 6.2 min. The half-life of bilirubin covalently bound to rat serum albumin was 1.9 to 2.1 days, identical to that of unmodified rat albumin. We conclude that bilirubin covalently attached to albumin is maintained in the circulation with the long half-life of albumin rather than the short half-life of bilirubin. Because albumin in humans has a half-life of 19 days, covalent attachment of bilirubin to human albumin could result in persistence of hyperbilirubinemia long after the resolution of disease.

Additional Keyphrases: animal models of human disease · radioassay · hepatobiliary disease

Bilirubin circulates in the plasma in several different chemical forms—as free bilirubin (bound reversibly by albumin), as a soluble monoglucuronide or diglucuronide conjugate, or covalently attached to albumin (1, 2). The last of these forms is found in patients with conjugated hyperbilirubinemia, such as obstruction, hepatitis, or cirrhosis, but not in patients with unconjugated hyperbilirubinemia, such as Gilbert’s disease, hemolytic disease, or physiological neonatal jaundice (3). The complex appears to arise from a spontaneous chemical reaction between albumin and bilirubin glucuronides, as shown by its occurrence exclusively in conjugated hyperbilirubinemia and by its spontaneous formation in vitro upon incubation of serum albumin with bilirubin glucuronides (4–6).

In patients treated for hepatobiliary disease or for biliary obstruction, the bilirubin concentration in serum is frequently monitored to confirm resolution of disease. Yet, in some patients, these concentrations remain increased even when other indicators suggest return of normal hepatobiliary function. If the various fractions of bilirubin are monitored, the covalent bilirubin–albumin complex becomes an increasing percentage of the total bilirubin as the disease itself resolves (5). Gautam and associates (6) suggested that hyperbilirubinemia probably persists because the metabolic half-life of bilirubin covalently attached to albumin is prolonged relative to that of the other forms of bilirubin, but they did not test this hypothesis.

Here we set out to determine how long the covalent bilirubin-albumin complexes were retained in the circulation relative to bilirubin itself and to albumin.

Materials and Methods

Preparation of Albums

Albumin was isolated from rat serum by affinity chromatography on Affigel Blue (Bio-Rad, Richmond, CA) and concanavalin A-Sepharose (Pharmacia, Piscataway, NJ) by the procedure of Feldhoff and Ledden (7). Monomer albumin was isolated by gel-filtration chromatography on G-100 Sephadex (Pharmacia).

Bilirubin (Sigma Chemical Co., St. Louis, MO) and [3H]bilirubin (Research Products International, Mount Prospect, IL) were confirmed to be >95% chemically or radiochemically bilirubin IX-α by thin-layer chromatography (8). We activated the bilirubin by reaction with Woodward’s Reagent K (N-ethyl-5-phenylisoxazolium-3'-sulfonate; Sigma Chemical Co.) and coupled this to albumin at molar ratios ranging from 1 to 3, by the procedure of Kuenzel et al. (9). The covalent bilirubin–albumin complexes were purified by passage through a column of human albumin immobilized on agarose to remove any uncoupled bilirubin and through a Sephadex G-100 column to isolate albumin monomer. The eluent was phosphate-buffered saline (0.1 mol NaCl, 16.2 mmol NaH2PO4, 3.7 mmol Na2HPO4 per liter), pH 7.4. Albumin immobilized on agarose was prepared as previously described (10).

To determine the amount of bilirubin covalently attached to albumin we measured the radioactivity associated with the complex formed from [3H]bilirubin (5 Ci/mol) or the absorbance at 453 nm for the complex prepared from nonradioactive bilirubin (molar absorptivity of bilirubin attached to rat albumin = 44 500 L·mol⁻¹·cm⁻¹). Albumin concentration was determined by the biuret reaction (11).

To determine whether the bilirubin moiety attached to albumin was the free acid form or the esterified form, we diazotized bilirubin–albumin with the diazonium salt of ethyl anthranilate and separated the cleaved dipyrrole azopigments by thin-layer chromatography on silica gel as described by Heirwegh et al. (12).

For iodination of albumin we used “Iodo-Beads” (Pierce Chemical Co., Rockford, IL) and Na131I or Na125I (New England Nuclear, Boston, MA). Less than 1 mol of iodine was incorporated per mol of albumin. Iodination of the covalent bilirubin–albumin complexes caused a loss of <12% of the intensity of the bilirubin absorption at 453 nm. Thus, the mild oxidizing conditions for iodination did not destroy the bilirubin label.

Turnover Studies

Male Sprague-Dawley rats were injected in a lateral tail vein with 300 μL of solution containing 10 mg of albumin (5 μCi of 125I-labeled albumin–bilirubin and 10 μCi of 131I-labeled albumin as a control for basal albumin turnover).
Blood from the opposite lateral tail vein was collected into heparinized tubes daily for seven days. The radioactivity in aliquots of plasma was counted with a Beckman 5500 gamma counter with 125I and 131I windows. This radioactivity was >95% precipitable with 50 g/L perchloric acid, showing that the radioactive iodide had remained attached to protein.

To verify that bilirubin was not cleaved from the covalent complex in the circulation, 1 μCi of [3H]bilirubin–albumin (15 mg of albumin) was injected into the lateral tail vein and monitored by serial plasma samples as described for the iodinated albumins. [3H]Bilirubin–albumin radioactivity was counted in a Beckman 7500 LS scintillation counter.

To determine the turnover of bilirubin in rats, we anesthetized them with sodium pentobarbital, passed a 10-cm catheter of no. 50 polyethylene tubing through the left carotid artery to the aortic arch, and clamped the catheter shut. We then injected 10 μCi of [3H]bilirubin in 300 μL of phosphate buffer containing 6.6 mg of rat serum albumin into a lateral tail vein (rat 1) or into the jugular vein (rat 2). Samples of blood were collected from the carotid artery at 30-s intervals by unclamping the catheter and collecting blood into heparinized tubes. The radioactivity in 25-μL aliquots of the resulting plasma was counted.

The half-life for each compound in the plasma was calculated from the linear regression analysis of a plot of log(difference/min) vs time.

Results

Characterization of the bilirubin–albumin complex. For this work we used synthetic covalent bilirubin–albumin complexes prepared from bilirubin esterified with Woodward's Reagent K rather than from bilirubin esterified with glucuronic acid, because we needed quantities of bilirubin covalently attached to albumin that are not readily prepared from bilirubin–glucuronide systems. To characterize our synthetic material, we cleaved the covalently attached bilirubin with the diazonium salt of ethyl anthranilic acid to produce two azodipyrroles, one of which remained covalently attached to albumin, the other being extracted into ethyl acetate. The extracted azodipyrrole was chromatographed by thin-layer chromatography and compared with similarly treated azodipyrroles generated from free bilirubin and from bilirubin esterified with Woodward's Reagent K. More than 70% of the azodipyrrole cleaved from the covalently attached bilirubin was found to be unconjugated; <30% was esterified with Woodward's Reagent K. Naturally occurring bilirubin–albumin complexes from rats yield between 50% and 80% unconjugated azodipyrrole and those from humans are >70% unconjugated azodipyrrole, with the remainder being mostly glucuronide conjugate (2, 13).

Therefore, the synthetic bilirubin–albumin used in this work, like the complexes formed in vivo, contained primarily unconjugated bilirubin coupled to albumin. Less than 30% of the synthetic bilirubin–albumin was esterified by Woodward's Reagent K (252 Da) and less than 30% of the naturally occurring bilirubin–albumin was esterified by glucuronic acid (194 Da).

Turnover studies. Turnover of 125I-labeled albumin–bilirubin was determined at the same time as the turnover of control 131I-labeled albumin in each of seven rats (Table 1). Both albumin alone and albumins with as many as 3 mol of covalently attached bilirubin per mole showed metabolic half-lives of 1.9 (0.2 SD) days. This value agrees well with the value of 1.8 (0.2 SD) days for rat albumin reported by Horbach et al. (14).

To assure ourselves that the attached bilirubin was not cleaved from albumin in the circulation, such that unmodified albumin would be compared with modified albumin, we also prepared [3H]bilirubin–albumin and injected this into two rats. The results were similar to the values determined with iodinated albumin species (Figure 1, Table 1). This demonstrates that the bilirubin label is not cleaved from circulating albumin but rather that the bilirubin is maintained in the serum covalently attached to albumin.

In contrast, when [3H]bilirubin was injected into the circulation in association with, but not covalently attached to, albumin it quickly disappeared from the circulation (Figure 2). As calculated with a single-compartment model, its half-life was only 6.2 (0.3 SD) min.

Discussion

We have shown that covalent bilirubin–albumin complexes remain in the circulation of rats with the two-day half-life of albumin rather than the 6-min half-life of unconjugated bilirubin. Therefore, the covalent attachment of bilirubin to albumin generates a form of bilirubin whose half-life is

Table 1. Metabolic Turnover of Albumin (A) and Covalent Bilirubin–Albumin (A-B) Complexes

<table>
<thead>
<tr>
<th>Rat wt, g</th>
<th>B/A, mol/mol</th>
<th>[125I]-A</th>
<th>[3H]-A-B</th>
<th>A-[3H]B</th>
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<tbody>
<tr>
<td>264</td>
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<td>2.2</td>
<td>1.9</td>
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</tr>
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<td>199</td>
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<td></td>
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<tr>
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<td>1</td>
<td>1.5</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
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<td>2</td>
<td>1.9</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
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<td>2.1</td>
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<tr>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
<td>265</td>
<td>1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.9 (0.2)</td>
<td>1.9 (0.1)</td>
<td>2.1 (0.1)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Plasma radioactivity after injection of 1 μCi of [3H]bilirubin–albumin into the tail vein of two male Sprague–Dawley rats (©) Rat 1, 265 g, t1/2 = 2.07 days. (A) Rat 2, 265 g, t1/2 = 2.15 days. The line reflects a composite linear regression fit of the data.
determined by the carrier protein. Extrapolating this metabolic behavior to humans, in whom the half-life of albumin is 19 days (15), covalent bilirubin–albumin complexes formed during hepatobiliary disorders could contribute to an increase of bilirubin in serum that may persist for several months.

In contrast, bilirubin itself is cleared rapidly from the circulation of both rats and humans. Similar to the observations depicted in Figure 2, Wolkoff et al. (16) reported that 38% of an injection of [3H]bilirubin in Sprague–Dawley rats was cleared by the liver within 15 min, and that by 15 min as much as 64% of the total injected bilirubin was conjugated to glucuronide by the liver. Studies with human subjects (17) showed that elimination of [14C]bilirubin over a 28-h period was better described by a three-compartment model than a simple one-compartment model but, in agreement with the results reported in this paper, most of the unconjugated bilirubin was eliminated from the circulation rapidly, with a half-life of <18 min.

Although it is unusual to find more than one molecule of bilirubin carried by an albumin molecule in icteric serum, albumin is believed to have three binding sites for bilirubin (18, 19). It is not known whether the albumin site that is occupied in spontaneously formed covalent complexes with bilirubin is the primary bilirubin-binding site or one of the secondary sites. Because the location of covalently bound bilirubin could affect the structure and catabolism of albumin, we prepared complexes of bilirubin–albumin with bilirubin attached to one, two or three sites. No differences were seen in turnover rates for the three different bilirubin complexes, indicating that the prolonged metabolic life of covalently bound bilirubin is not a function of the particular site on albumin to which it is attached.

No metabolic consequence of the prolonged presence of covalent bilirubin–albumin complexes in the circulation has been documented. However, covalent attachment of bilirubin to albumin does interfere with dye-binding assays for quantifying albumin, presumably by irreversible occupancy of a potential dye-binding site by a bilirubin molecule, resulting in an underestimation of albumin concentration (20). Because various ligands, such as sulfonamides, are transported on the bilirubin-binding site of albumin (21), their patterns of distribution and turnover may be altered by the limited availability of binding sites on covalent bilirubin–albumin complexes.

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References