Role of apolipoprotein D in the transport of bilirubin in plasma

WOLFRAM GOESSLING1 AND STEPHEN D. ZUCKER2
1Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115; and 2Division of Digestive Diseases, University of Cincinnati Medical Center, Cincinnati, Ohio 45267

Received 9 June 1999; accepted in final form 18 March 2000

Goessling, Wolfram, and Stephen D. Zucker. Role of apolipoprotein D in the transport of bilirubin in plasma. Am J Physiol Gastrointest Liver Physiol 279: G356–G365, 2000.—Apolipoprotein D (apo D) is a 30-kDa glycoprotein of unknown function that is associated with high-density lipoproteins (HDL). Because unconjugated bilirubin has been shown to bind apo D with a 0.8:1 stoichiometry, we examined the contribution of this protein to transport of bilirubin in human plasma. Density gradient centrifugation analysis using physiological concentrations of [14C]bilirubin reveals that 9% of unconjugated bilirubin is associated with HDL, with the remaining pigment bound primarily to serum proteins (i.e., albumin). The percentage of total plasma bilirubin bound to HDL was found to increase proportionally with bilirubin concentration. Affinity of human apo D for bilirubin was determined by steady-state fluorescence quenching, with Scatchard analysis demonstrating a single binding site for apo D for this bile pigment. The half-time for dissociation from apo D was measured at 5.4 ± 1.0 s. Incorporation of apo D into phosphatidylcholine vesicles had no effect on the affinity of the protein for bilirubin. Using stopped-flow techniques, the first-order rate constant for bilirubin dissociation from apo D was measured at 5.4 ± 1.0 s−1 (half-time = 129 ms). Our findings indicate that HDL is the principal nonalbumin carrier of bilirubin in human plasma and further support the proposition that the affinity of HDL for bilirubin is primarily the result of binding to apo D. High-density lipoproteins; human serum albumin; dissociation rate; cholesterol; cholic acid

UNCONJUGATED BILIRUBIN (UCB), the principal product of heme catabolism, is efficiently cleared from the circulation by the liver. In patients with impaired hepatic function, plasma bilirubin levels can increase 50-fold, producing yellow discoloration of the skin, sclerae, and mucous membranes. Bennhold (3) first postulated more than 60 years ago that bilirubin, which exhibits minimal aqueous solubility at physiological pH, is transported in the blood bound to serum albumin. Electrophoretic analyses of human plasma by Ostrow et al. (30) subsequently confirmed that circulating bilirubin is associated with albumin. However, despite the high affinity of human serum albumin (HSA) for bilirubin (6, 24, 35), the notion that albumin is vital for bilirubin transport was dispelled by the observation that individuals with analbuminemia, a rare genetic disorder associated with negligible serum albumin concentrations, exhibit normal bilirubin clearance (15, 42). It is notable that, in both analbuminemic humans (15) and rats (39), bilirubin is associated predominantly with high-density lipoproteins (HDL). This finding is not entirely unexpected, since Cooke and Roberts (14) previously have shown that a proportion of serum bilirubin is complexed with β-lipoproteins at physiologically relevant concentrations. Although bilirubin is known to bind to phospholipids (23, 28, 41), it remains unclear why this pigment preferentially associates with HDL as opposed to other lipoprotein classes (e.g., low-density lipoproteins).

Apolipoprotein D (apo D), a 30-kDa glycoprotein of unknown function, comprises roughly 1–2% of HDL (26). apo D is structurally unrelated to other apolipoproteins, exhibiting sequence homology with lipocalins, a family of proteins that bind small hydrophobic ligands (44). On the basis of molecular modeling analyses, Peitsch and Boguski (31) hypothesized that apo D preferentially binds heme-related compounds. These authors went on to show that bilirubin, at supraphysiological concentrations, binds to purified apo D with a stoichiometry of 0.8 moles of bilirubin per mole of protein. To extend these observations and verify whether apo D confers the binding specificity of HDL for bilirubin, we examined the contribution of lipoproteins to the binding and transport of bilirubin in human plasma and further characterized the affinity of apo D for this bile pigment.

EXPERIMENTAL PROCEDURES

Materials. Bilirubin IXα, the physiological isomer of UCB, was obtained from Porphyrin Products (Logan, UT). For all apo D binding analyses, bilirubin was further purified by phase extraction according to the method of McDonagh and Assisi (27). δ-[4-14C]aminolevulinic acid hydrochloride was purchased from NEN Life Sciences (Boston, MA). Essentially fatty acid-free HSA was obtained from Sigma Chemical (St. Louis, MO). All glassware was washed in chloroform before use to eliminate potential lipid contamination.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Preparation of radiolabeled UCB. UCB was radiolabeled biosynthetically by infusing the metabolic precursor, 8-[4-14C]aminolevulinic acid, into male Sprague-Dawley rats (16, 29). [14C]bilirubin was isolated from a 6-h collection of bile by gel filtration of glucuronides, precipitation with lead acetate, extraction with chloroform, and subsequent recrystallization (16). The specific activity of the bilirubin prepared in this manner ranged between 35 and 75 Ci/mol.

Determination of the binding distribution of bilirubin in human plasma. Blood from nonfasted healthy volunteers was drawn into tubes containing 5% (vol/ vol) 0.2 M EDTA, and plasma was isolated by centrifugation at 265 g for 15 min. The phlebotomy protocol and consent procedure were approved by the Brigham and Women’s Hospital Human Research Committee (Protocol no. 96-07907). Plasma samples were treated with ascorbic acid (6 mM) to prevent bilirubin oxidation and with NaN3 (0.01% wt/vol) to inhibit microbial growth. Because of poor aqueous solubility at physiological pH (6), UCB was solubilized in 0.1 M potassium phosphate (pH 7.4) (51). In a standard experiment, a 3 mM stock solution of UCB was dissolved in alkaline buffer and spiked with enough [14C]bilirubin to produce 10,000–12,000 dpm/μl. A small (18 μl) aliquot of the bilirubin stock solution was added to 3.6 ml of plasma, producing a final bilirubin concentration of 15 μM. Although this manipulation caused minimal alteration of the pH of the plasma sample (± 0.02 pH units), an equal volume (18 μl) of 0.1 M potassium phosphate (pH 1.9) was immediately added to correct the pH to baseline.

Samples of human plasma were incubated with radiolabeled bilirubin for 15 min at room temperature and then adjusted to a density of 1.21 g/ml with solid KBr (0.325 g/ml). Following centrifugation at 265 g for 15 min, the phlebotomy protocol and consent procedure were approved by the Brigham and Women’s Hospital Human Research Committee (Protocol no. 96-07907). Plasma samples were treated with ascorbic acid (6 mM) to prevent bilirubin oxidation and with NaN3 (0.01% wt/vol) to inhibit microbial growth. Because of poor aqueous solubility at physiological pH (6), UCB was solubilized in 0.1 M potassium phosphate (pH 7.4) (51). In a standard experiment, a 3 mM stock solution of UCB was dissolved in alkaline buffer and spiked with enough [14C]bilirubin to produce 10,000–12,000 dpm/μl. A small (18 μl) aliquot of the bilirubin stock solution was added to 3.6 ml of plasma, producing a final bilirubin concentration of 15 μM. Although this manipulation caused minimal alteration of the pH of the plasma sample (± 0.02 pH units), an equal volume (18 μl) of 0.1 M potassium phosphate (pH 1.9) was immediately added to correct the pH to baseline.

Samples of human plasma were incubated with radiolabeled bilirubin for 15 min at room temperature and then adjusted to a density of 1.21 g/ml with solid KBr (0.325 g/ml) to facilitate resolution of plasma lipoprotein [very low-density lipoprotein, low-density lipoprotein (LDL), HDL] and protein components by density gradient centrifugation (11). Following centrifugation (197,000 g × 48 h) at 17°C, sequential 200-μl fractions were harvested and 50-μl aliquots were diluted into 5 ml of Ecoscint (National Diagnostics, Atlanta, GA) and counted. The average recovery was 99.8 ± 0.3% of bilirubin added counts. Fraction density was determined using a Mettler/Paradrome DMA 45 density meter (Anton Paar, Graz, Austria). Protein was quantified by the method of Lowry et al. (11, 25), phospholipid by the assay technique of Bartlett (2), and cholesterol by the enzymatic method of Allain et al. (1).

Purification of human HDL and apo D. apo D was purified from human plasma according to the method of McConathy and Alaupovic (26). One liter of plasma was adjusted to a density of 1.12 g/ml. Following repeat centrifugation at 140,000 g for 3 h at 17°C, sequential 200-μl fractions were harvested and 50-μl aliquots were diluted into 5 ml of Ecoscint (National Diagnostics, Atlanta, GA) and counted. The average recovery was 99.8 ± 0.3% of bilirubin added counts. Fraction density was determined using a Mettler/Paradrome DMA 45 density meter (Anton Paar, Graz, Austria). Protein was quantified by the method of Lowry et al. (11, 25), phospholipid by the assay technique of Bartlett (2), and cholesterol by the enzymatic method of Allain et al. (1).

Preparation of apo D affinity for bilirubin. A variety of fluorescence quenching analyses were performed to facilitate the determination of the affinity of apo D for UCB. For each of these studies, commercial bilirubin was further purified by phase extraction (27) to avoid potential competition with contaminating lipid species. Serial 2-μl aliquots of purified UCB (500 μM in DMSO) were added to a stirred cuvette containing 3 ml of a 10 μM solution of apo D in 0.1 M potassium phosphate (pH 7.4). The steady-state fluorescence (excitation: 280 nm, emission: 352 nm) of apo D at 25°C was recorded following the addition of each aliquot using an Amino-Bowman II fluorescence spectrophotometer. All readings were corrected for the added volume of DMSO and for inner filter effects resulting from bilirubin absorption (51). The affinity constant (Kd) was determined by Scatchard analysis, according to the method of Levine (24).

The affinity of apo D for bilirubin was also determined by measuring the equilibrium distribution of UCB between apo D and HSA. For these studies, UCB (0.25 mM) was solubilized in 50 mM potassium phosphate (pH 12) and 10-μl aliquots were added to 1 ml of 0.1 M potassium phosphate (pH 7.4) containing varying ratios of apo D to HSA. Partitioning was quantified by recording steady-state bilirubin fluorescence (excitation: 467 nm, emission: 525 nm) and fitting the data to the expression

\[
\frac{I_{\text{HSA}} - I_{\text{apo D}}}{I_{\text{apo D}}} = 1 + K_{\text{apo D}}^{\text{HSA}} \frac{[\text{apo D}]}{[\text{HSA}]}
\]

where Iapo D and I_HSA indicate the fluorescence intensity of bilirubin in the presence of apo D or HSA alone, Iapo D is the observed bilirubin fluorescence at a defined molar ratio of apo D/[HSA], Kd is the affinity constant, and [apo D] and [HSA] are the concentrations of apo D and HSA, respectively. The total protein concentration ([apo D] + [HSA]) was maintained constant to control for inner filter effects, and all data were corrected for intrinsic protein fluorescence. Based on Eq. 1, the slope of a plot of \(I_{\text{apo D}} \cdot I_{\text{HSA}}^{-1} - I_{\text{apo D}}^{-1}\) vs. \([\text{apo D}]/[\text{HSA}]\) provides a measure of the relative affinity \(K_{\text{apo D}}^{\text{HSA}}/K_{\text{apo D}}\) of the proteins for bilirubin. Samples were maintained in the dark and only briefly exposed to light for all fluorescence measurements to minimize the risk of bilirubin photodegradation. We further measured bilirubin binding by the fluorimetric titration of apo D (10 μM) in 0.1 M potassium phosphate (pH 7.4) with increasing concentrations of bilirubin (excitation: 467 nm, emission: 525 nm). With this approach, the affinity of apo D for bilirubin was derived from a linear least-square plot, according to the method of Cogan et al. (13).

Preparation of phospholipid vesicles. Small unilamellar vesicles were prepared by sonication according to the method of DiCorleto and Zilversmit (17). A chloroform solution of egg phosphatidylcholine (Lipid Products, Surrey, UK) was evaporated to dryness under argon atmosphere, solubilized in diethyl ether, and then reevaporated to form a uniform film. The lipids were desiccated overnight under vacuum to remove all traces of solvent and then suspended in 0.1 M potassium phosphate (pH 7.4). The lipid suspension was sonicated under argon atmosphere in a bath sonicator (Lab- toratory Supplies, Hicksville, NY) until clear. apo D was incorporated into small unilamellar vesicles by sonication, according to the method of Levine (24).

Stopped-flow analysis of bilirubin dissociation. The rate of bilirubin dissociation from isolated HDL, HSA, and apo D was determined from the time-dependent changes in intrinsic protein fluorescence (excitation: 280 nm, emission: 360 nm), reflecting bilirubin transfer from the donor particle to a large molar excess of small unilamellar phosphatidylcholine acceptor vesicles (49). Studies of bilirubin dissociation from HSA and apo D employed an Amino-Bowman II fluorescence spectrophotometer equipped with an SLM-Aminco MilliFlow reactor (mixing time: 20 ms) using a 320-nm cut-off emission filter to minimize light-scattering effects. Because of the
rapid rate of bilirubin dissociation from HDL, an Applied Photophysics (Leatherhead, UK) fluorescence spectrophotometer with SPF-17 stopped-flow device (mixing time: 0.7 ms) was utilized. The rate constant for bilirubin dissociation from HDL, HSA, and apo D was determined by fitting the observed fluorescence intensities to both single and double exponential functions, with fit quality assessed by regression analysis of variance (51). In the case of a double exponential fit, an average rate \( k_{av} \) for bilirubin dissociation was calculated by the expression (38)

\[
k_{av} = \frac{k_{fast} A_{fast} + k_{slow} A_{slow}}{A_{fast} + A_{slow}}
\]

where \( A \) is the amplitude and \( k \) the rate constant for the fast and slow components.

RESULTS

**Distribution of bilirubin in human plasma.** The binding distribution of \([^{14}C]\)bilirubin in human plasma was determined by KBr density gradient centrifugation. This technique was found to provide excellent resolution of plasma LDL, HDL, and protein fractions (Fig. 1), the latter consisting primarily of HSA (8). The results of experiments analyzing the distribution of physiological (15 \( \mu M \)) concentrations of UCB in human plasma are displayed in Fig. 2. We found that 89.2 \( \pm \) 0.4\% of added \([^{14}C]\)UCB was associated with the protein fraction of human plasma, whereas 9.1 \( \pm \) 0.4\% was bound to HDL and 1.5 \( \pm \) 0.1\% to LDL. Since UCB is associated with serum HDL in analbuminemic rats (19), we also examined the distribution of \([^{14}C]\)UCB in rat plasma and found it to be similar to that in human plasma, with 87.7 \( \pm \) 0.4\%, 10.9 \( \pm \) 0.3\%, and 1.4 \( \pm \) 0.1\% bound to protein, HDL, and LDL, respectively.

To confirm the binding of bilirubin to HDL, we preincubated a suspension of HDL (isolated from human plasma) or a solution of HSA with \([^{14}C]\)UCB and then performed density gradient centrifugation (Fig. 2, inset). The principal peaks of radioactivity coincide with
those of plasma HDL and protein, respectively. We postulate that the smaller high-density peak observed in the HDL-bound bilirubin sample represents unbound (aggregated) bilirubin. This hypothesis is supported by experiments in which centrifugation of [14C]UCB was performed in the absence of carrier particles (Fig. 2, inset). Further validation of the centrifugation technique was achieved by examining the distribution of [14C]cholic acid and [14C]cholesterol in human plasma (Fig. 3). Our results correlate closely with previous reports (8, 21, 34) and confirm that the observed plasma distribution patterns are ligand specific.

Effect of concentration and pH on the distribution of bilirubin in plasma. Since serum bilirubin levels are markedly elevated under certain pathological conditions, we utilized density gradient centrifugation to examine the effect of bilirubin concentration on its plasma distribution. In these experiments, [14C]UCB (15–900 μM) was added directly to samples of human plasma (Fig. 4). The percentage of UCB associated with the protein fraction of plasma was found to decrease with increasing bilirubin concentration (Fig. 4, inset). This decline in the percentage of albumin-associated bilirubin coincides with an increase in the relative amount of bilirubin bound to HDL, which rose to a maximum of 19% at 900 μM UCB. On the basis of the observation that the risk of bilirubin neurotoxicity is increased in the setting of acidosis (7), we also determined the effect of pH on the binding distribution of UCB in human plasma. Plasma pH was adjusted between 6.8 and 8.0 before the addition of radiolabeled bilirubin. Plasma distribution of cholic acid and cholesterol. Density gradient centrifugation was performed on samples of human plasma preincubated with 1 μM [14C]cholic acid (■) or 2 nM [14C]cholesterol (●) under the same conditions as outlined in Fig. 2.

Fig. 3. Plasma distribution of cholic acid and cholesterol. Density gradient centrifugation was performed on samples of human plasma preincubated with 1 μM [14C]cholic acid (■) or 2 nM [14C]cholesterol (●) under the same conditions as outlined in Fig. 2.

Fig. 4. Distribution of bilirubin in human plasma: influence of bilirubin concentration. Samples of human plasma were preincubated with [14C]UCB at concentrations of 15 μM (●), 120 μM (■), and 900 μM (▲), corresponding to bilirubin-to-albumin molar ratios of 1:60, 1:5, and 1.5:1, respectively. The plasma samples were subjected to density gradient centrifugation for 48 h at 17°C, and 200-μl aliquots were sequentially harvested and counted. In the inset, the percentages of total counts associated with HDL (●) and HSA (▲) are plotted as functions of the bilirubin:albumin molar ratio.
bilirubin (15 μM), and density gradient centrifugation subsequently was performed. No significant effect of pH on the distribution of UCB was observed (data not shown).

**Determination of apo D binding affinity for bilirubin.** To examine whether the selective binding of bilirubin by HDL is due to the presence of apo D, we isolated apo D from human plasma (Fig. 5) and prepared rabbit anti-apo D antiserum. Samples of human and rat plasma were then separated by density gradient centrifugation, and the various lipoprotein and protein fractions were harvested, subjected to SDS-PAGE, and blotted with anti-apo D antiserum (Fig. 6). The highest concentrations of apo D were identified in the HDL fractions of both human and rat plasma. The affinity of purified apo D for UCB was measured using resonance energy transfer techniques. The addition of increasing concentrations of UCB to a solution of apo D causes progressive quenching of the intrinsic tryptophan fluorescence of the protein (Fig. 7). Scatchard analysis of the quenching curve (Fig. 7, inset) demonstrates a single, high-affinity bilirubin binding site, with a $K_a$ of $2.6 \pm 0.5 \times 10^7$ M$^{-1}$.

To confirm these findings, the affinity of apo D for UCB was also calculated from the equilibrium binding distribution of bilirubin between apo D and HSA ($K_a^{apo D}$) as measured by bilirubin steady-state fluorescence (Fig. 8). The ratio of the affinity constants ($K_a^{apo D}/K_a^{HSA}$) is derived from the slope of a plot of bilirubin fluorescence intensity vs. the apo D:HSA molar ratio (Fig. 8, inset). With the use of a consensus value for $K_a^{HSA}$ of $1.1 \times 10^8$ M$^{-1}$ (6, 12, 24, 46), the affinity constant of apo D for UCB is calculated to be $1.2 \pm 0.3 \times 10^7$ M$^{-1}$, which is similar to the results obtained by Scatchard analysis.

When a solution of apo D is titrated with increasing concentrations of UCB, an approximately linear rise in bilirubin fluorescence intensity is observed, with saturation occurring as the molar ratio of bilirubin:apo D approaches 1:1 (Fig. 9). An apparent dissociation constant ($K_d$) of $25 \pm 19$ nM was derived from a linear least-square plot of the data (Fig. 9), using the method of Cogan et al. (13). The calculated $K_d$ corresponds to a $K_a$ of $4.0 \times 10^7$ M$^{-1}$, which closely correlates with affinity constants obtained by the previously outlined methods. The calculated number of binding sites ($n$) is $0.8 \pm 0.2$, consistent with a 1:1 binding stoichiometry.

Although apo D has no identifiable membrane-spanning domains (31, 44), the protein is associated primarily with lipid structures (i.e., cell membranes, lipoproteins) in vivo (47). For this reason, we examined whether the affinity of the protein for bilirubin is altered by a lipid environment. Following the incorporation of apo D into small unilamellar phosphatidylcholine vesicles, we determined the binding affinity for UCB by competitive binding against HSA. The measured $K_a$ for membrane-associated apo D (2.5 $\times 10^7$ M$^{-1}$) is not significantly different from values obtained for free apo D, whereas the affinity constant for phosphatidylcholine (PC) vesicles alone is $\sim 0.08\%$ that of

---

**Fig. 5. SDS-PAGE of human apolipoprotein D (apo D).** A 10-μg sample of apo D isolated from human plasma was subjected to SDS-PAGE on a 14% polyacrylamide gel and stained with Coomassie blue.
Stopped-flow analysis of bilirubin dissociation from HDL and apo D. There is evidence that bilirubin clearance from the plasma is limited by the rate of dissociation from serum albumin (45, 50). To assess whether HDL-bound bilirubin may be cleared more efficiently than albumin-bound bilirubin, we compared the rate of dissociation of UCB from isolated human HDL with that from HSA using stopped-flow techniques. The average rate of UCB dissociation from HDL (Eq. 2) was found to be \(240\)-fold faster than from HSA (Fig. 10), suggesting that HDL is the more efficient bilirubin donor. Regression analysis of the fluorescence tracings reveals that bilirubin dissociation from HDL is best described by a double exponential function (Fig. 11). The rate constant of 215 \(\pm\) 14 s\(^{-1}\) [half-time \((t_{1/2})\) = 3.2 \(\pm\) 0.2 ms] for the fast component of dissociation is identical to previously reported off rates for UCB from small unilamellar phospholipid vesicles (51). On the basis of these findings, we postulate that the fast component of dissociation from HDL represents bilirubin solvation from surface phospholipids. We further propose that the slow component reflects bilirubin dissociation from apo D. The 1:1 ratio of the amplitudes of the fast and slow components of bilirubin dissociation suggests that approximately half of HDL-bound bilirubin is associated with apo D. The dissociation of UCB from purified apo D was best described by a single exponential function (Fig. 11, inset), with a first-order rate constant of 5.4 \(\pm\) 1.1 s\(^{-1}\) (\(t_{1/2}\) = 12.9 \(\pm\) 2.6 ms).

DISCUSSION

The present study demonstrates that HDL is the principal carrier of non-albumin-bound bilirubin in plasma, consistent with previous observations in analbuminemic humans (4) and rats (19). Although the amount of UCB partitioning into HDL under normal physiological conditions is only 9% of total plasma levels, we show that the relative contribution of HDL to UCB binding increases at higher molar ratios of bilirubin to albumin, as can occur in various disease states. Our findings are concordant with prior work demonstrating the formation of \(\beta\)-lipoprotein-bilirubin...
complexes in human plasma when bilirubin concentrations approach the saturation limit of albumin (14). The substantial bilirubin carrying capacity of HDL is reflected in the normal bilirubin transport and clearance observed in analbuminemic individuals (4, 20, 43).

From the results of plasma distribution studies, we conclude that the affinity of HDL for UCB exceeds that of LDL by an order of magnitude, a phenomenon that cannot be explained by lipid binding alone (23, 28, 41). The observed second-order dissociation kinetics suggest the existence of two distinct populations of bilirubin associated with the HDL particle. We postulate that the fast component reflects bilirubin bound to surface phospholipids, a hypothesis that is supported by the similarity in the off-rate constant to that for bilirubin solvation from small unilamellar vesicles (51). We further propose that the slow phase of bilirubin dissociation from HDL is the result of binding to apo D, as evidenced by the close correspondence of the rate constant with that for bilirubin solvation from purified apo D. Taking into account the measured binding affinity of apo D and phospholipids for bilirubin, as well as the bilirubin binding distribution in plasma, our data correlate closely with the affinity of HDL for UCB estimated from studies of analbuminemic rats (39).

apo D is structurally unrelated to other apolipoproteins, and the precise function of this protein remains obscure. It is classified as a member of the lipocalin family of binding proteins (36), a characteristic feature of which is the presence of a binding pocket formed by eight antiparallel strands. Insecticyanin, a lipocalin isolated from the tobacco hornworm, *Manduca sexta*,

---

**Fig. 8.** Binding distribution of UCB between HSA and apo D. The intrinsic fluorescence of 2.5 μM UCB was measured in the presence of varying concentrations of HSA and apo D, maintaining the total protein concentration constant at 24 μM. Each point represents the mean ± SD of 3 experiments performed at 25°C ($r^2 = 0.994$). Inset: inverse of the fractional fluorescence is plotted against the molar ratio of apo D:HSA. The slope of the plot (0.11 ± 0.01) reflects the ratio of the association constants ($K_{apoD} / K_{HSA}$). [apo D] and [HSA], concentrations of apo D and HSA, respectively; $I_{HSA}$, $I_{apoD}$, and $I_{obs}$, fluorescence intensity of bilirubin in the presence of HSA alone, apo D alone, or observed fluorescence intensity at a defined molar ratio of [apo D] to [HSA], respectively.

---

**Fig. 9.** Affinity of apo D for bilirubin as determined by fluorimetric titration. A: steady-state bilirubin fluorescence was recorded at 25°C following the addition of increasing concentrations of UCB to a solution of 10 μM apo D (•) or to buffer alone (■). Data generated in the presence of apo D after correction for the contribution of free bilirubin fluorescence are shown as ▲. B: linear least-square plot of the data ($r^2 = 0.989$), where $P_0$ is the total protein concentration, $R_0$ is the total bilirubin concentration, and $a$ reflects the fraction of free binding sites on the apo D molecule (13).
binds biliverdin IXα with high specificity and is believed to be important for protective coloration (18, 32). On the basis of the high degree of homology between insecticyanin and apo D, including the topology of the binding pocket, Peitsch and Boguski (31) originally proposed that apo D is capable of binding heme-related compounds. These authors also showed that bilirubin IXα binds to apo D at a molar ratio of 0.8:1, albeit at supersaturating bilirubin concentrations. Our studies extend the observations of these investigators, confirming the high-affinity binding of UCB by apo D ($K_a \approx 3 \times 10^7 M^{-1}$) and further support a role for this protein in bilirubin transport. It is notable that the affinity of apo D for UCB is similar to that measured for lipocalin-type prostaglandin D synthase (5). Because prostaglandin D synthase is the most abundant protein in human cerebrospinal fluid, it has been proposed that this molecule may act as a scavenger for harmful hydrophobic compounds (5). On the basis of the high levels of expression of apo D in the brain (44), it is conceivable that apo D may serve a similar function.

There is evidence suggesting that solvation from HSA is the rate-limiting step in the hepatocellular uptake of UCB (48, 50). Since bilirubin dissociation from HDL (and apo D) is significantly faster than from HSA, it appears that HDL is the more proficient bilirubin donor. The increased rate of appearance of circulating bilirubin in the bile of analbuminemic rats compared with control animals supports this contention (39). It is notable that the risk of bilirubin-induced neurotoxicity (kernicterus) in newborns correlates most closely with the concentration of nonalbumin-bound bilirubin in the plasma (10). The observation that HDL is the principal nonalbumin carrier of UCB suggests that HDL-bound bilirubin may be an important mediator of bilirubin toxicity, perhaps as a result of increased availability for uptake into the central nervous system. This hypothesis is supported by the higher levels of UCB in the brains of analbuminemic rats infused with HDL-bound vs. albumin-bound bilirubin (40). Although it has been reported that the risk of kernicterus is increased in the setting of acidosis (37), we found no effect of pH on the binding distribution of bilirubin in human plasma.
Serum albumin concentrations in the early neonatal period are dependent on gestational age, varying from a mean of 1.9 g/dl before 30 wk gestation to 3.1 g/dl at term (9). One study revealed a mean serum albumin level of 2.5 g/dl (range: 0.8–4.0 g/dl) for infants with gestational ages ranging from 26 to 42 wk (mean: 33 wk) admitted to the neonatal intensive care unit (33). Although serum levels of apo D also appear to correlate with gestational age, variations are much less pronounced compared with albumin, with a mean value of 3.7 ± 1.4 mg/dl at birth (22). Compared with average adult values for serum albumin (4.2 g/dl) and apo D (12 mg/dl), neonatal serum has a significantly lower total bilirubin binding capacity. On the basis of the results of our centrifugation studies, the reduced serum albumin levels in newborns would be expected to result in higher levels of HDL-bound bilirubin, potentially increasing the availability of bilirubin for uptake into the brain. Support for this hypothesis is derived from the work of Cooke and Roberts (14), who demonstrated the presence of β-lipoprotein-bilirubin complexes in the serum of a jaundiced neonate but not in normal controls.

We gratefully acknowledge the technical assistance of Drs. Alison Hoppin, Xiaoyang Qi, and Gregory A. Grabowski in the performance of fluorescence analyses. We also thank Dr. J. Donald Ostrow for helpful advice regarding bilirubin purification and Drs. Richard M. Green, John L. Gollan, and Martin C. Carey for their valuable comments and criticism. Preliminary reports of this work have been published in abstract form (Hoppin AG et al. Gastroenterology 108: A1086, 1995; Goessling W and Zucker SD, Hepatology 26: 385A, 1997).

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-51679 (S. D. Zucker), a Charles H. Hood Foundation Child Health Research Award (S. D. Zucker), a postdoctoral award from the BASF-Foundation, Germany (W. Goessling), and the Alfried Krupp von Bohlen und Halbach-Stiftung, Germany (W. Goessling).

REFERENCES