# Fatty Acid Enhancement of the Quantum Yield for the Formation of Lumirubin from Bilirubin Bound to Human Albumin[1]

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**ABSTRACT. During phototherapy for neonatal jaundice, bilirubin is converted into a number of different polar photoproducts. Because fatty acids are bound to human albumin *in vivo* and have been shown to affect the binding of bilirubin to albumin, we examined the effect of various fatty acids on the photochemistry of albumin-bound bilirubin. Fatty acids of carbon chain length of 10 or more were found to increase by as much as 3-fold the quantum yield for formation of lumirubin, an intramolecularly cyclized isomer of bilirubin. The binding of these same fatty acids was found to affect visible absorption and circular dichroic spectra of the bound bilirubin, a finding previously interpreted to be the result of a conformational change in the bilirubin. The increased quantum yield for the formation of lumirubin appears to be the result of an allosterically induced conformational change in bilirubin which is bound to albumin. (*Pediatr Res* 21: 530–533, 1987)**

The use of light in the treatment of neonatal jaundice was first described nearly 30 yr ago (1). While little has changed over the past three decades in the way in which phototherapy is administered to jaundiced infants, our understanding of how phototherapy works has been revised and refined many times. Recent evidence from a number of clinical studies indicates that the elimination of bilirubin during phototherapy is principally the result of the formation and excretion of a polar structural isomer of bilirubin, called lumirubin (2–5). The importance of lumirubin to pigment elimination during phototherapy is surprising because *in vitro* experiments have shown that the rate of lumirubin formation is slow; however, these measurements have been performed using bilirubin bound to human serum albumin in the absence of fatty acids, which are bound to albumin *in vivo* (6, 7). We report herein that the binding of long-chain fatty acids to bilirubin-albumin complexes *in vitro* markedly enhances the rate of formation of lumirubin.

## METHODS

All work with bilirubin was performed under photographic safe lights. Crystalline analytical grade bilirubin (Serva Fine Biochemicals, Garden City Park, NY), purified as previously described (8), was dissolved in 0.1 M NaOH and added to a final concentration of 30 $\mu$M to a solution of 60 $\mu$M human albumin

(essentially fatty acid free, Sigma Chemical, St. Louis, MO), 0.15 M NaCl, 1 mM Na$_2$EDTA, 0.05 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4. Hexanoic, octanoic, decanoic, lauric, myristic, and stearic acids were obtained from Aldrich Chemical Co. (Milwaukee, WI); palmitic, oleic, and linoleic acids were obtained from Sigma Chemical Co. All were free acids of the highest purity available. The fatty acids were dissolved in methanol containing equimolar NaOH and aliquots were dried under Ar to a film on glass tubes (9). The bilirubin-albumin solution was added and the solutions were mixed either by vortexing or stirring until optically clear. Three concentrations of fatty acids were studied: 1.0, 2.5, and 5.0 equivalents per mole of albumin. Three ml of each solution was placed in a polystyrene cuvette and sealed. The cuvettes were placed on the perimeter of a turntable in holders which masked all but the front surface. The samples were irradiated for 30 min with white light from a bank of eight Duro-Test Vita Lites, while the turntable was rotated at 50 rpm, ensuring that both the fatty acid-containing and reference (no fatty acid) solutions received equal incident irradiation. Under the conditions used, approximately 6% of the bilirubin in the reference solution (with no fatty acid) was converted to lumirubin. After the irradiation, the samples were analyzed by high-pressure liquid chromatography as previously described (10). The amount of lumirubin formed in each sample, determined from the integrated area under the lumirubin peak on the chromatogram, was divided by the amount of lumirubin formed in simultaneously irradiated reference solutions.

Absorbance and circular dichroism spectra were obtained on unirradiated samples in 1-cm quartz cuvettes. The absorbance spectra were measured in a Cary 210 dual-beam spectrophotometer. Circular dichroism spectra were measured in a Jasco model J-40A spectrometer, which was calibrated with (+)-10-camphor sulfonic acid.

## RESULTS

For each fatty acid studied, three measurements were made to determine the effects of binding of the acid to bilirubin:albumin complexes: the rate of lumirubin formation, the visible absorption spectrum, and the circular dichroism spectrum. The rate of lumirubin formation from bilirubin bound to human albumin showed a progressive increase with the addition of up to five equivalents of fatty acid of carbon chain length of 10 or more (Table 1). Above five equivalents of fatty acids, further increases in the rate of lumirubin production were seen; however, because of the displacement of bilirubin from albumin at these higher concentrations of fatty acids, the results were variable (results not shown). Hexanoic and octanoic acids, unlike the longer chain fatty acids, produced only a slight increase in the rate of lumirubin production which did not show a trend with concentration (Table 1).

Table 1. *Effect of fatty acids on rate of lumirubin formation**

| Fatty acid:HSA molar ratio | Hexanoic C-6 | Octanoic C-8 | Decanoic C-10 | Lauric C-12 | Myristic C-14 | Palmitic C-16 | Steric C-18 | Oleic C-18 *cis* 9† | Linoleic C-18 *cis, cis* 9, 12† |
|---|---|---|---|---|---|---|---|---|---|
| 0.0‡ | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 1.0 | 1.09 | 1.09 | 1.16 | 1.77 | 1.32 | 1.19 | 1.34 | 1.36 | 1.46 |
| | (0.02)§ | (0.01) | (0.01) | (0.01) | (0.04) | (0.02) | (0.03) | (0.01) | (0.01) |
| 2.5 | 1.14 | 1.13 | 1.43 | 2.74 | 2.24 | 2.16 | 2.28 | 2.09 | 2.06 |
| | (0.02) | (0.02) | (0.01) | (0.02) | (0.02) | (0.13) | (0.03) | (0.02) | (0.06) |
| 5.0 | 1.05 | 1.09 | 2.01 | 3.58 | 3.46 | 3.21 | ¶ | 2.99 | 3.12 |
| | (0.05) | (0.03) | (0.04) | (0.14) | (0.07) | (0.12) | | (0.02) | (0.01) |

* Values are the amount of lumirubin formed in sample with added fatty acid divided by the amount of lumirubin formed in a simultaneously irradiated reference solution with no fatty acid.

† Position and configuration of double bonds in unsaturated fatty acids.

‡ Reference solution containing no added fatty acid.

§ Values in parentheses are the deviation of duplicate experiments.

¶ Five equivalents of stearic acid would not go into solution.

The binding of fatty acids of carbon chain length of 10 or more to bilirubin-albumin complexes led to a progressive shift in the absorption maximum to longer wavelength (bathochromic shift) and a progressive increase in the strength of the absorption maximum (hyperchromicity). This is illustrated in Figure 1 for the binding of lauric acid to bilirubin-albumin complexes. The binding of the other long-chain fatty acids produced qualitatively similar shifts in the bilirubin absorption spectrum, and all spectra had an isosbestic point at 442 ± 2 nm. Absorption spectra of samples containing hexanoic or octanoic acid were indistinguishable from the spectrum for bilirubin:albumin alone. Bilirubin contains no asymmetric carbons and is not optically active when dissolved in organic or aqueous solution. However, when bound to albumin, bilirubin has a strong bisignate circular dichroism signal which is the result of the dissymmetric binding of bilirubin in a right-handed helical conformation (11). The binding of the long-chain fatty acids also affected the position and intensities of the circular dichroism bands of bilirubin. This is illustrated in Figure 2 for the binding of lauric acid to bilirubin:albumin complexes. The binding of the other long-chain fatty acids produced qualitatively similar changes in the circular dichroism spectrum of bilirubin. The circular dichroism spectra of samples containing hexanoic or octanoic acid were indistinguishable from the spectrum for bilirubin:albumin alone. Thus, except for hexanoic and octanoic acids, the binding of fatty acids produced changes in the rate of lumirubin formation, the visible absorption spectrum, and the circular dichroism spectrum.

## DISCUSSION

During phototherapy, light energy is used to convert unconjugated bilirubin from an insoluble, unexcretable form to more water-soluble photoproducts. The major photoproducts formed *in vivo* are configurational isomers, mainly 4Z,15E-bilirubin, and a structural isomer, lumirubin. In human infants, the more rapidly formed configurational isomers are excreted only very slowly (4). Evidence from a number of clinical studies indicates that the rapid excretion of the more slowly formed lumirubin is the principal route of bilirubin elimination during phototherapy (2, 4, 5).

Fatty acids bind to human albumin at sites which are distinct from the primary bilirubin binding site (12). The binding of fatty acids has an effect on the circular dichroism spectrum of albumin-bound bilirubin (Fig. 3) which has been reported previously (9, 13). This change in the circular dichroism spectrum has been interpreted to be the result of a conformational change of the bilirubin molecule about the central methylene bridge such that the dihedral angle between the two dipyrrolic chromophores of bilirubin is altered (14). This effect of fatty acid binding on the conformation of albumin-bound bilirubin is shown schemati-
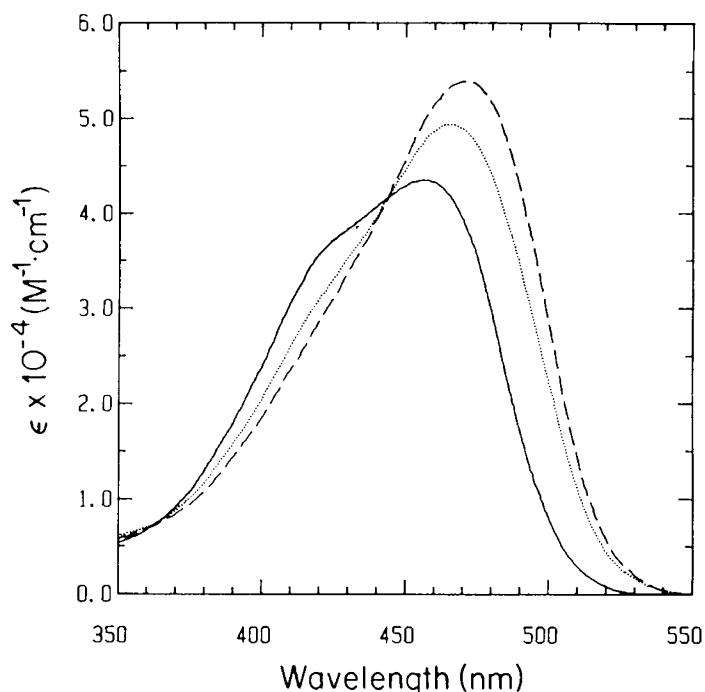


Fig. 1. The effect of lauric acid binding to human albumin on absorption spectrum of bilirubin. The molar extinction coefficient, ε, is plotted as a function of wavelength. No lauric acid (*solid line*); 2.5 equivalents of lauric acid per mole of human albumin (*dotted line*); 5.0 equivalents of lauric acid per mole of human albumin (*dashed line*).

cally in Figure 3. Because the photochemistry of bilirubin is sensitive to the surrounding environment, we examined the effect of fatty acid binding on the rate of lumirubin formation. The results in Table 1 demonstrate that those fatty acids which bind to albumin and produce a conformational change in bilirubin enhance the rate of formation of lumirubin.

Two factors contribute to the increased rate of lumirubin formation. One factor is that with the addition of fatty acids, bilirubin bound to albumin absorbs light more strongly (Fig. 1). Therefore, with equal incident irradiation, as used in these experiments, the bilirubin:albumin solutions containing fatty acids will absorb more photons. However, the hyperchromicity produced by fatty acid binding is small when compared to the increased rate of lumirubin production. For example, the increased light absorption in the samples containing 5 equivalents of lauric acid was less than 18% [estimated by numerical integration of the overlap between the emission spectrum of the light
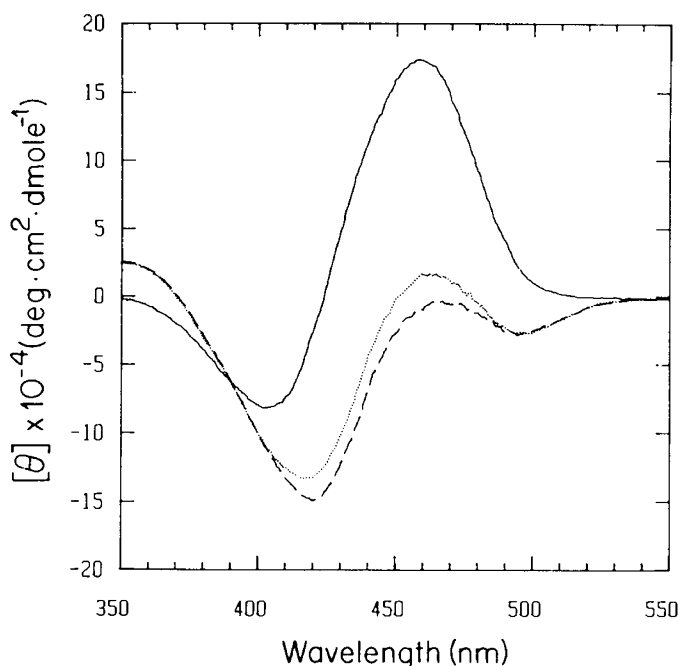
Fig. 2. The effect of lauric acid binding to human albumin on circular dichroism spectrum of bilirubin. Molar ellipticity ($\theta$) is plotted as a function of wavelength. No lauric acid (*solid line*); 2.5 equivalents of lauric acid per mole of human albumin (*dotted line*); 5.0 equivalents of lauric acid per mole of human albumin (*dashed line*).



Fig. 3. Schematic representation of the binding of bilirubin to albumin in the absence (*left*) and presence (*right*) of fatty acids. The two dipyrrolic chromophores of bilirubin, represented as *dashed ovals*, are bound to separate domains on albumin, represented as the larger, *hatched ovals*. In the absence of fatty acids, the angle between the two chromophores is 109° [11]. Fatty acids, represented as an oval hydrophilic end and a long hydrophobic tail, bind to two other domains on albumin, causing a conformational change in the protein and an increase in the dihedral angle between the two chromophores of bilirubin [modified from Brodersen [24]].

source [15] and the absorption spectra for samples containing 0 and 5 equivalents of lauric acid], whereas the increase in lumirubin production is more than 300%. The major contributor to the enhanced production of lumirubin with increasing fatty acid:albumin molar ratio is an increase in the quantum yield for the formation of lumirubin. An albumin-bound bilirubin molecule that absorbs a photon is more likely to form lumirubin if fatty acids are also bound to the albumin. This is presumably the result of the conformational change in the bilirubin molecule produced by the fatty acid binding (Fig. 3), although proof of this relationship will require further work.

Recent studies have provided evidence that during phototherapy the formation of lumirubin is the step which determines the rate at which bilirubin is eliminated [5, 16]. The importance of lumirubin in the therapeutic efficacy of phototherapy has been somewhat surprising to many investigators because of the very low quantum yield for the formation of lumirubin, especially when compared to the quantum yield for the $Z$ to $E$ configurational isomerization (*i.e.*, 0.0012 *versus* 0.20) [17] (Malhotra V, Ennever JE, unpublished data). However, all previous *in vitro* studies of the rate of lumirubin formation have used bilirubin bound to albumin which has been defatted. Our results suggest that the rate of formation of lumirubin *in vivo*, where the molar ratio of fatty acid:albumin is generally between 0.5 and 3.0 [19], is higher than that predicted based upon *in vitro* studies with defatted albumin.

Our results suggest that the rate of lumirubin production may be faster and phototherapy perhaps more effective if the fatty acid:albumin ratio were maintained at the higher range of normal. A study is in progress to determine if the rate of formation of lumirubin in jaundiced infants receiving phototherapy can be correlated with fatty acid:albumin molar ratios. It is well known that very high levels of fatty acids can result in the displacement of bilirubin from albumin both *in vitro* and *in vivo* [20–23]. Thus, the administration of fatty acid emulsions to jaundiced infants is potentially dangerous. The allosteric conformational change in bilirubin bound to albumin which we have associated with an increased rate of lumirubin formation is a more subtle
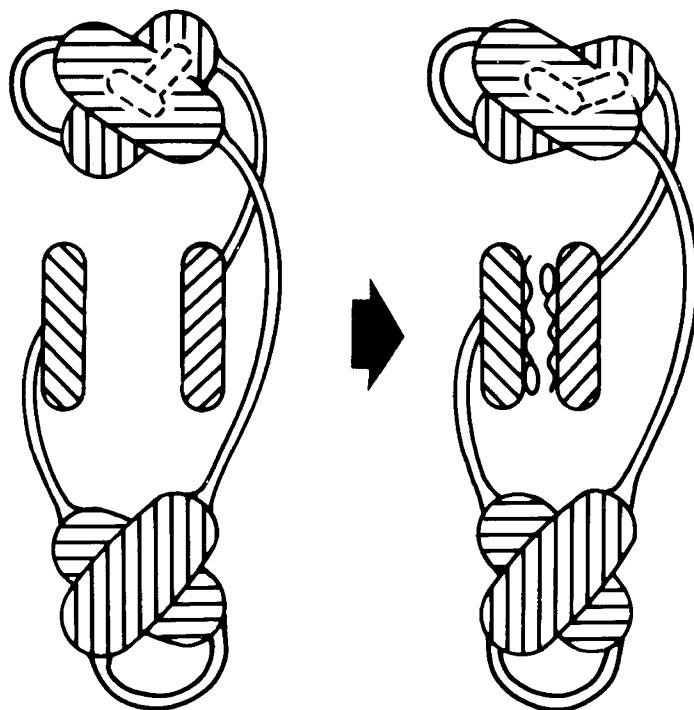
change than displacement and occurs at lower fatty acid:albumin molar ratios.

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# Announcement

## Symposium on Developmental Gastroenterology

The Ohio State University and the Children's Hospital Research Foundation, Columbus, OH will hold the First Wexner Symposium at the Hyatt Regency Hotel from September 13 through September 15, 1987. The symposium has been approved for 10 hours of CME credit.

*Invited faculty:* Vay Liang W. Go, M.D.; Michael D. Gershon, M.D.; Alan Lucas, M.B., B. Chir, M.R.C.P.; William Balisteri, M.D.; Susan J. Henning, Ph.D.; Otakar Koldovsky, M.D., Ph.D.; Zane Cohen, M.D., F.R.C.S.(C), F.A.C.S.; W. Allan Walker, M.D.

*Major topics:* bacterial colonization of the gut; gut hormone responses; action of pancreatic polypeptide; signaling and neural messages in the gut; endogenous intestinal hormones; hormones in milk; glucose polymer assimilation; newborn colonic function; intestinal vascular regulation; immune function in the gut; and immunology of intestinal transplantation.

*For further information contact:* Howard R. Sloan, M.D., Ph.D., Children's Hospital, 205 Ross Hall, 700 Children's Drive, Columbus, OH 43205 or call 614-461-2212.