Kinetics of Bilirubin Binding to Bovine Serum Albumin and the Effects of Palmitate*

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The binding of bilirubin to bovine serum albumin has been determined to be a second order process, first order in each reactant. Formation of the bilirubin·albumin complex was observed as an increase in absorbance at 489 nm by stopped flow spectrophotometry. The effect of long chain fatty acid on the binding of bilirubin was investigated at palmitic acid levels of 0 to 7 mol/mol of albumin at pH 7.4. The second order rate constant is \( 1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) when the palmitate/albumin ratio is less than 5 and drops to \( 0.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) when the palmitate/albumin ratio reaches 6. The equilibrium association constant for the bilirubin·albumin complex is \( 5.5 \times 10^6 \text{ M}^{-1} \) when the palmitate/albumin ratio is less than 1, \( 18 \times 10^6 \text{ M}^{-1} \) when the palmitate/albumin ratio is between 1 and 5, and \( 3 \times 10^6 \text{ M}^{-1} \) when the palmitate/albumin ratio is greater than 5. The mechanism of the effect of palmitate varies with the total palmitate level. At low molar ratios, addition of palmitate has no effect on the association rate but decreases the dissociation rate. At high molar ratios, addition of palmitate decreases the association rate and increases the dissociation rate. It is suggested that palmitate affects the ability of the protein to undergo the conformational changes needed to accommodate bilirubin.

Serum albumin serves as a carrier protein for a wide variety of substances including fatty acids, bilirubin, tryptophan, cystine, and various drugs and hormones (1, 2). Since many of these substances are present in serum at the same time, the effects of one ligand on the binding and transport of another are of practical concern.

Much attention has focused on the displacement of bilirubin from albumin since the clinical consequence of increased levels of free bilirubin in the newborn—kernicterus—is well known (3). Sulfonamides, salicylates, and fatty acids have all been reported to displace bilirubin from serum albumin (4-6). Results from in vitro displacement studies, however, have not always agreed with clinical observations. For example, Ode11 et al. (7) observed that low levels of salicylates produce significant displacement of bilirubin in the sera of jaundiced patients but little displacement from comparable bilirubin/albumin solutions.

To date, competitive binding studies on the displacement of bilirubin from albumin have utilized systems at equilibrium. Since circulating proteins and ligands constitute a dynamic system, rates of binding and release of ligands may be as important as the position of equilibrium. The work reported here was undertaken to extend our understanding of ligand- ligand interactions through observation of kinetics as well as equilibrium effects of the displacement of bilirubin from albumin by a long chain fatty acid. A preliminary report of this work has been presented (8).

**EXPERIMENTAL PROCEDURES**

Materials

Armour crystalline bovine serum albumin was defatted at pH 3 on an AG1-X8 ion exchange resin following the procedure of Scheider and Fuller (9). Concentration of albumin was determined by \( A_{280} = 6.6 \) (10).

Palmitic acid was added in one of two ways. (a) A solution of palmitic acid in 1-propanol was added dropwise to a stirred solution of albumin at room temperature. Total 1-propanol added never exceeded 0.2% of the total volume. At low molar ratios (<3) of palmitate to albumin, the solution remained cloudy even after 4 days at 37°. After filtration of the turbid solution, the fatty acid content of the albumin never exceeded a molar ratio of 7.1. (b) A heptane solution of palmitic acid was spread over the surface of a glass container and the heptane was evaporated. A solution of albumin was added and equilibrated with gentle rocking for 24 h at 37°. The maximum fatty acid content achieved by this method was 6.5:1 when a 50-molar excess of palmitate was present initially. The maximum palmitate to albumin molar ratio achieved by Spector and Hoak using palmitate-coated Celite to load bovine serum albumin was 7.1 (11).

The fatty acid content of the albumin was determined in one of two ways: (a) a colorimetric determination of the copper salts of palmitate by the procedure of Mikac-Devic (12); (b) use of low specific activity \(^{14} \text{C}\) palmitate for refatting followed by scintillation counting. Results from the two procedures were in good agreement.

Bilirubin (Nutritional Biochemicals) exhibited a molar absorptivity in chloroform of 61,350 at 453 nm and was used without further purification since it met the standard of purity that molar absorptivity in chloroform be at least 60,700 ± 1,600 at 453 nm (13). Horseradish peroxidase was Sigma type II (Sigma Chemical Co.) and \(^{14} \text{C}\) palmitic acid was an Amersham/Searle product.

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* This study was supported by United States Public Health Service Research Grant HL-22751 and the Stephen C. Clark Research Fund of The Mary Imogene Bassett Hospital. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(Received for publication, May 31, 1977)
All work was carried out in phosphate-buffered saline, NaCl/P,
(0.1 M NaCl, 16.2 mM NaHPO₄, and 3.75 mM NaH₂PO₄, pH 7.4) at 37°C unless otherwise indicated.

Methods

Spectra were recorded on a Hitachi Coleman 124 spectrophotometer with cell compartment maintained at 37°C. Kinetic studies were carried out on a Durrum model D-110 stopped flow spectrophotometer with a Beckman DU monochrometer and monitored on a 5103 Tektronix oscilloscope system with a D15 storage unit. Dead time for the instrument is less than 1 ms.

Kinetic Studies—For determination of the forward rate constant kₕ, solutions of albumin (2 mg/ml) were prepared in degassed solutions of 0.2 M NaCl, 32.4 mM Na₂HPO₄, and 7.5 mM NaH₂PO₄. Bilirubin (150 PM) was dissolved in degassed 5 mM NaOH containing 1 mM EDTA; pH was 9.5 ± 0.3. The absorption spectrum of bilirubin was recorded immediately after its preparation and again after all kinetic experiments had been completed. During the 3- to 4-h interval, less than a 1% change was observed for absorbance at 440 or 480 nm. The concentration of bilirubin was determined using a molar absorptivity of 47,509 M⁻¹ cm⁻¹ at 440 nm (14). The solution of bilirubin was stored in an opaque container; the reservoir syringe of bilirubin was wrapped in aluminum foil. Subdued lighting was used during the preparation of bilirubin solutions and during the experimental procedures.

Initial absorbance was determined as the sum of the absorbance for each reactant solution mixed with an equal volume of water by stopped flow spectrophotometry. The output to the oscilloscope was offset to compensate for the initial absorbance and the y axis was adjusted to display the absorbance change over the full vertical grid. Equal volumes of albumin and bilirubin were mixed and the absorbance change observed as a function of time. Each pair of solutions was observed at least six times. Absorbance time points were read directly from the oscilloscope and treated as described under “Results.” After 3 min, a final absorbance reading was recorded.

For determination of the reaction order a similar procedure was followed for a series of albumin and bilirubin solutions of different concentrations. The value of t₁/₂ was recorded as time corresponding to an observed absorbance of ½ (Aₘₐₓ + Aₐₘₜ).

Equilibrium Studies—The concentration of free bilirubin in equilibrium with albumin bound bilirubin was determined from the rate of oxidation of free bilirubin by peroxide-peroxidase. Solutions of bilirubin (150 PM) were prepared in dilute NaOH containing 1 mM EDTA with final pH 9.5 ± 0.3. Between 2.5 and 15 nmol of bilirubin was added to 1 ml of NaCl/P₄ containing 15 nmol of albumin in a 1-cm cuvette. A spectrum was recorded between 500 and 360 nm. Aliquots of horseradish peroxidase (0.06 mg/ml in NaCl/P₄) and hydrogen peroxide (0.05% in water) were added and the absorbance at λₘₐₓ was recorded for a period of 3 to 5 min. Oxidation of bound bilirubin was assumed to occur at a negligible rate. The equilibrium association constant was determined from the slope of a plot of the reciprocal of the initial rate against the reciprocal of peroxidase concentration. The kinetics of this system are explained in detail by Jacobsen and Wennberg (14).

Results

Spectral Properties—The shift in the absorption spectrum of bilirubin upon binding to crystalline albumin is demonstrated in Fig. 1. The absorbance maximum is shifted from 438 nm for free bilirubin in aqueous solution to 470 nm for bilirubin bound to albumin and the molar absorptivity is increased about 20%. The maximum color change occurs at 450 nm, demonstrated by the difference spectrum between a solution of free bilirubin and the same concentration of bilirubin in aqueous solution to 470 nm for bilirubin in the presence of excess albumin (Fig. 1, inset).

The effect of palmitate on the absorption spectrum of bilirubin in the presence of albumin is shown in Fig. 2. In the absence of fatty acid, the absorbance maximum occurs at 463 nm and the molar absorptivity is 63,600 at a palmitate to albumin ratio between 2 and 4. At higher palmitate levels, λₘₐₓ and molar absorptivity decrease. This same pattern was reported by Woolley and Hunter (15) for bilirubin/bovine serum albumin in the presence of oleate, although they observed a more marked decrease in absorptivity for fatty acid/albumin ratios above 5.

Binding was observed by the increase in absorbance at 480 nm.
nm when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not when bilirubin was added to albumin. Although this is not the wavelength of maximum color change for all albumin samples in this study, substantial color change took place even for those samples with low \( \lambda_{\text{max}} \) and \( \epsilon \). A value of \( \epsilon_{\text{ave}} \) was determined for each albumin by adding a known amount of bilirubin to an excess of protein. Values of \( \epsilon_{\text{ave}} \) ranged from 48,200 to 60,900 for bilirubin-albumin complexes compared to \( \epsilon_{\text{ave}} \) of 24,000 for free bilirubin.

The stoichiometry of bilirubin binding to crystalline albumin is illustrated by spectrophotometric titration (Fig. 3). The rate of color change with increasing bilirubin content shows two distinct breaks at mole ratios of 1:1 and 3:1 consistent with three bilirubin binding sites. The slope of the line beyond mole ratio 3:1 is similar to that of the lower curve — free bilirubin in the absence of albumin. The pattern is consistent with one strong site and two weak sites reported by Jacobsen for human albumin (16). The absorption spectrum of bilirubin bound to one of these secondary sites, shown in Fig. 1, was obtained as the difference spectrum of solutions of 1:1 and 2:1 bilirubin to albumin. This spectrum is very similar to that for bilirubin in the presence of a COOH-terminal fragment of bovine serum albumin, residues 306 to 581, which has a weak binding site for bilirubin (17).

**Rate Expression** — The reaction was determined to obey the following rate expression,

\[
\text{rate} = k_{1}[\text{albumin}]^m[Bilirubin]^n
\]

where \( n = 2 \) and \( m = 1 \). The rate observed was the appearance of complex as evidenced by an increase in absorption at 480 nm with time.

The overall reaction order, \( n \), was determined to be second order by use of the half-life method. Bilirubin and albumin were initially at the same concentration so that a simplified rate law (Equation 2) applied and a plot of \( \log t_{1/2} \) against \( \log C_0 \) (initial concentration) gave a straight line with slope \( (1 - n) \).

\[
\log t_{1/2} = (1 - n) \log C_0 + \text{constant}
\]

The half-time of the reaction between albumin and bilirubin ranged from 2.4 to 135 ms over a concentration range of 29.4 to 4.4 \( \mu \text{M} \). The data were plotted according to Equation 2 and the slope was determined by linear regression to be \( -0.953 \).

The overall reaction order would then be 1.953 which was considered to be a reaction order of 2.

The reaction order with respect to bilirubin was also determined using the half-life method. The initial concentration of albumin was 90 \( \mu \text{M} \) and the bilirubin concentration ranged from 5 to 18 \( \mu \text{M} \), making the reaction appear to be zero order in albumin. The half-time, 38 ± 4 ms, did not vary with the bilirubin concentration indicating a reaction first order in bilirubin.

Since the reaction was demonstrated to be second order overall and first order in bilirubin, the order with respect to albumin must also be first order. This could not be confirmed by observing the dependence of \( t_{1/2} \) on albumin in the presence of excess bilirubin since a substantial color change occurs due to the binding of bilirubin at secondary sites.

**Determination of \( k_{1} \)** — Since the reaction between bilirubin and albumin follows second order kinetics, first order in each reactant, the integrated form of the rate law is

\[
k_{1}t = \frac{1}{a - b} \ln \frac{b(a - x)}{a(b - x)}
\]

where \( a \) and \( b \) are the initial concentrations of albumin and bilirubin, respectively, and \( x \) is the concentration of complex at time \( t \). Rearrangement of Equation 3 to the following expression illustrates that a plot of the natural logarithm of the ratio of initial concentration of albumin to unreacted bilirubin at time \( t \) against \( t \) gives a straight line of slope \( (a - b)k_1 \), and intercept of natural logarithm of the ratio of initial concentrations.

\[
\ln \frac{(a - x)}{(b - x)} = (a - b)k_1t + \ln \frac{a}{b}
\]

Observed absorbance value at time \( t \) was converted to \( \ln((a - x)/(b - x)) \) using the following relationships.

\[
b - x = A_\infty - \frac{A - A_\infty}{2\epsilon_{\text{ave}}}
\]

\[
a - x = a - [b - (b - x)]
\]

where \( A_\infty \) = final absorbance at \( t = \infty \) in a 2-cm cell; \( A \) = absorbance at \( t \) in a 2-cm cell; \( \epsilon_{\text{ave}} = \epsilon_{\text{ave}}(\text{complex}) - \epsilon_{\text{ave}}(\text{free bilirubin}) \) (complex) was determined individually for each BSA sample; \( a \) = initial concentration of albumin; \( b \) = initial concentration of bilirubin. When \( \ln((a - x)/(b - x)) \) was plotted against \( t \), a straight line was observed (Fig. 4). Linear regression analysis was used to determine the slope, intercept, and correlation coefficient. The correlation coefficients ranged from 0.990 to 0.999. The good fit of the data to Equation 4 provides additional support that the rate expression in Equation 1 is valid.

**Temperature Dependence** — The forward rate constant \( k_1 \) increases with increasing temperature. Values of \( k_1 \) were determined to be \( 0.134 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) at 7°, \( 0.469 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) at 25°, \( 1.20 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) at 37°, and 3.38 \( \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) at 52°.

The dependence of \( k_1 \) on temperature is a reflection of activation energy as expressed by the Arrhenius equation

\[
\ln k = \frac{E_a}{R} \left( \frac{1}{T} \right) + \text{constant}
\]

where \( E_a \) is the activation energy. A plot of \( \ln k \) against \( 1/T \) results in a straight line (\( r^2 = 0.999 \)) whose slope corresponds to an activation energy of 12.9 kcal/mol. Typically, \( E_a \) for a simple second order process ranges from 10 to 50 kcal/mol (18). \( k_1 \) and \( K_1 \) as a Function of Palmitate Concentration — Crystalline albumin was defatted and refatted with palmitic acid and correlation coefficient. The correlation coefficients ranged from 0.990 to 0.999. The good fit of the data to Equation 4 provides additional support that the rate expression in Equation 1 is valid.
acid to produce a series of albumins with fatty acid content ranging from 0 to 7. The method of preparation did not appear to affect the bilirubin binding properties of refatted albumin.

No significant difference in the forward rate constant was observed until the palmitate/albumin ratio exceeded 5, with fatty acid content above 5 mol/mol being too great to be accounted for by a 2% increase in free bilirubin. The alterations in the visible spectrum of bilirubin is shifted to higher wavelength and is intensified upon binding to albumin. The position and shape of this curve vary with the fatty acid content of the albumin. Maximum intensity and high $\lambda_{max}$ are associated with those albumin samples with the greatest affinity for bilirubin. The decreased intensity and shift to lower wavelength in the cases of defatted albumin and albumin with fatty acid content above 5 mol/mol are too great to be attributed to a simple composite spectrum of bound and free bilirubin. For example, 7 $\mu$M bilirubin in the presence of 15 $\mu$M albumin would be 99.3% bound if $K_s$ were 20 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ and 97.3% bound if $K_s$ were 5.5 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$.

**Table 1**  
Rate and equilibrium constants for bilirubin binding to albumin at various palmitate levels

<table>
<thead>
<tr>
<th>mol palmitate/mol albumin</th>
<th>$k_1 \times 10^{-6}$</th>
<th>$K_s \times 10^{-6}$</th>
<th>$k_1 - k_s K_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M$^{-1}$ s$^{-1}$</td>
<td>M$^{-6}$</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>0</td>
<td>1.43 ± 0.3e</td>
<td>5.5 ± 1.0e</td>
<td>0.26 ± 0.07e</td>
</tr>
<tr>
<td>0.5-0.99</td>
<td>1.56 ± 0.3</td>
<td>8.5 ± 0.9</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>1-1.99</td>
<td>1.59 ± 0.2</td>
<td>18.1 ± 1.0</td>
<td>0.088 ± 0.02</td>
</tr>
<tr>
<td>2-2.99</td>
<td>1.63 ± 0.3</td>
<td>17.4 ± 1.5</td>
<td>0.094 ± 0.02</td>
</tr>
<tr>
<td>3-3.99</td>
<td>1.75 ± 0.4</td>
<td>18.0 ± 2.5</td>
<td>0.087 ± 0.03</td>
</tr>
<tr>
<td>4-4.99</td>
<td>1.44 ± 0.2</td>
<td>14.6 ± 1.3</td>
<td>0.088 ± 0.02</td>
</tr>
<tr>
<td>5-5.99</td>
<td>0.63 ± 0.1</td>
<td>4.8 ± 0.6</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>6-6.99</td>
<td>0.48 ± 0.1</td>
<td>3.0 ± 0.4</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>0°</td>
<td>1.40 ± 0.3</td>
<td>21.0 ± 0.8</td>
<td>0.067 ± 0.02</td>
</tr>
</tbody>
</table>

* Since the numbers of individual albumin samples included within each palmitate range vary, the standard error of the mean is used as a measure of precision.

* Crystalline bovine serum albumin.

**DISCUSSION**

The reaction between bilirubin and albumin was established to be second order-first order in each reactant. This is in good agreement with the continuous flow studies reported by Faerch and Jacobsen (19) who also monitored color change of bilirubin and reported a second order process with a forward rate constant of 0.9 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ at 36°C. These results are in disagreement with Chen (20) who reported that binding monitored by quenching of protein fluorescence was too fast to measure by stopped flow. He observed a first order development of bilirubin fluorescence and a slower secondary quenching of protein fluorescence and proposed that binding occurs as a complex, multistage process. Some evidence for multistage binding was also reported by Faerch and Jacobsen who observed that the increase in absorbance toward the end of the reaction did not occur as rapidly as their initial rate data predicted. In the present study a similar trend was observed. The calculated forward rate constant tended to be higher when determined from short reaction times and lower when determined from longer reaction times, resulting in a very small difference in calculated values of $k_1$, e.g. 1.14 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ from 0 to 100 ms and 0.91 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ from 0 to 200 ms. The phenomenon is more noticeable in the $y$ intercept of Fig. 4 which should be 0.8 as calculated from the logarithm of the ratio of initial concentrations, but is in fact 1.0, indicating that the early reaction produced a more intense color change than the later reaction profile reflects. This would be compatible with an initial second order binding process followed by a relaxation or a small migration of bilirubin which results in a slight color loss. Such a migration of bilirubin along a binding crevice was suggested by Chen to explain the observed secondary quenching of the protein tryptophan fluorescence.

It is difficult to explain why Chen did not see a second order process. Although he used higher concentrations of albumin and bilirubin than in the present studies, which would result in a faster reaction, and an instrument with a relatively long dead time (10 ms), the second order rate data predict a reaction half-time of greater than 13 ms for his experimental conditions. Perhaps the mechanism for fluorescent quenching is not the same as that for producing the "bound bilirubin" absorbance spectrum.

The visible spectrum of bilirubin is shifted to higher wavelength and is intensified upon binding to albumin. The position and shape of this curve vary with the palmitate content of the albumin. Maximum intensity and high $\lambda_{max}$ are associated with those albumin samples with the greatest affinity for bilirubin. The decreased intensity and shift to lower wavelength in the cases of defatted albumin and albumin with fatty acid content above 5 mol/mol are too great to be attributed to a simple composite spectrum of bound and free bilirubin. For example, 7 $\mu$M bilirubin in the presence of 15 $\mu$M albumin would be 99.3% bound if $K_s$ were 20 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ and 97.3% bound if $K_s$ were 5.5 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$. Defatted albumin shows a much greater spectral change than could be accounted for by a 2% increase in free bilirubin. The alterations in the spectra of bilirubin bound to albumin containing varying amounts of palmitate must, therefore, reflect differences in the local environment of the bound pigment rather than varying contributions of unbound pigment to the absorption spectra.
Bilirubin, upon binding to bovine serum albumin, develops a strong CD spectrum. Woolley and Hunter (15) reported that addition of oleate caused marked changes in this spectrum under conditions in which little or no bilirubin was displaced from albumin. They considered three possible explanations for the alterations in CD spectra. (a) Binding of fatty acid in distant locations caused a conformational change in the protein. (b) Fatty acid displaced bilirubin to secondary sites. (c) Locally bound fatty acid interacted directly with the bilirubin site or with bilirubin itself.

The effect of palmitate on the rates of association and dissociation (as calculated from $K_a$ and $k_d$) highlights the complexity of the binding process. Equilibrium association is weakened both by the absence of fatty acid and the presence of high levels of fatty acid but the mechanisms are different. With defatted albumin the weakened binding is the result of an increased rate of dissociation. In the presence of high levels of palmitate, the weakened binding is primarily a result of a decreased rate of association although the rate of dissociation is elevated as well.

The association of fatty acids with albumin is known to cause configurational changes which increase the stability of the protein molecule. Defatted albums are more unstable on storage than albums containing fatty acid (21). Differences have also been noted in the ultraviolet absorption spectra (22) and the isoelectric points (23) of albumin in the presence and absence of fatty acids. The configurational change in albumin upon binding of fatty acid is undoubtedly also responsible for the spectral differences and dissociation rate differences observed for bilirubin binding. It is challenging to visualize how a pre-existing configurational change due to bound fatty acid would affect the rate of dissociation but not that of association for bilirubin binding. It is tempting to consider that palmitate in some way renders the protein more flexible and that the initial binding is followed by a secondary relaxation process which moves bilirubin deeper into a binding crevice. Consequently, the dissociation process would occur less facilely under the influence of fatty acid than in its absence. Patterns of $K_a$ and $k_d$, with $t$, however, indicate bilirubin binding to defatted albumin undergoes the same type of relaxation process as with other albumin samples.

The altered rates of association and dissociation at high levels of fatty acid most likely reflect a different mechanism than that for defatted albumin—perhaps a direct interaction between the two ligands. The effect of palmitate is unlikely to be a stoichiometric displacement of bilirubin since secondary fatty acid binding is of much lower affinity than primary bilirubin binding (24) and palmitate at secondary sites would not be effective in displacing bilirubin. The primary, strong palmitate sites are thought to be located outside the primary bilirubin binding region (17) and binding at these sites does not adversely affect bilirubin binding (Table I).

In conclusion, I would like to suggest that the first and third hypotheses of Woolley and Hunter (15) might be applied to defatted and highly fatted albumin, respectively. The initial fatty acid molecules bind in distant locations and cause conformational changes in the protein which enhance the protein's ability to accommodate bilirubin. The secondary fatty acid molecules (5th to 7th sites) bind in locations close to the bilirubin site and directly affect the stability and flexibility of that region, reflected in both association and dissociation processes.

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