Effect of Serum Dilution on Apparent Unbound Bilirubin Concentration as Measured by the Peroxidase Method

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I studied the effects of serum dilution and oxidation of albumin-bound bilirubin on the accuracy of results by the peroxidase method for assessing bilirubin–albumin binding. The apparent concentration of unbound bilirubin decreases with dilution of bilirubin-enriched serum or defatted-albumin solutions, the effect being more marked with serum. The decrease in apparent unbound bilirubin does not appear to be due to slow oxidation of albumin-bound bilirubin. Instead, the bilirubin–albumin complex has a much lower apparent dissociation rate constant \(K_{-1}\) in serum \(0.0033 \pm 0.0002 \text{ s}^{-1}\) than in solutions of defatted-albumin \((0.017 \pm 0.006) \text{ s}^{-1}\), causing the dissociation of the complex to be rate-limiting when serum is analyzed at the currently recommended 40-fold dilution and peroxidase concentration \((0.11 \mu\text{mol/L})\). In addition, dilution appears to enhance bilirubin binding by serum but not by defatted-albumin solutions. Decreasing the serum dilution and peroxidase concentration may significantly improve the accuracy of the peroxidase test. The actual correlation between clinical bilirubin toxicity and specific unbound bilirubin concentrations, however, remains to be determined.

Additional Keyphrases: bilirubin toxicity, mechanism of... newborns - bound and unbound bilirubin

The peroxidase method for determining the apparent unbound bilirubin concentration (AUBC)\(^1\) in sera from jaundiced infants is a sensitive, precise, and now automated test currently in use in several clinical laboratories (7). Although correlating well with other methods in which bilirubin–albumin binding is evaluated (2), the accuracy of the peroxidase test has been difficult to assess because no other bilirubin–albumin binding test directly measures the concentration of unbound bilirubin.

Accurate determination of the unbound bilirubin is important, not only in the clinical application of the test but also in establishing the correct quantitative relationship between bilirubin binding in serum and the uptake and toxicity of bilirubin in tissues (3).

Serum is ordinarily diluted about 40-fold before analysis by the peroxidase method, reducing the amount of serum required for the test. Although the dilution decreases the concentrations of both total and albumin-bound bilirubin by 40-fold, the concurrent change in unbound bilirubin concentration should be negligible, because bilirubin binds to at least two albumin binding sites and the equilibrium association binding constants are high (4–6).

Jacobsen and Pedders (7), however, using undiluted serum, reported much higher AUBC's by the peroxidase test than were later observed by Jacobsen and Wennberg (8), who diluted the serum 40-fold. The former authors detected no oxidation of albumin-bound bilirubin in serum, but the latter authors reported a decrease in reaction velocity (and thus a decrease in AUBC) with increasing serum dilution. The decrease in AUBC was attributed to slow oxidation of albumin-bound bilirubin, and they suggested a correction factor. Brodersen et al. (9) found that horseradish peroxidase and peroxide appeared to oxidize albumin-bound bilirubin in bilirubin-enriched solutions of Cohn Fraction V albumin, but that the apparent oxidation of bound bilirubin disappeared after the albumin was defatted.

In an effort to clarify the effects of serum dilution and oxidation of albumin-bound bilirubin on the peroxidase analysis of bilirubin–albumin binding, I undertook the following studies.

Materials and Methods

Reagents and Solutions

Phosphate buffer, 0.055 mol/L, pH 7.4, containing 1 mmol of disodium ethylenediaminetetraacetate (EDTA) was used for all solutions unless otherwise indicated.

Bilirubin, horseradish peroxidase (HRP, EC 1.11.1.7) Type I, and defatted human serum albumin were obtained from Sigma Chemical Co., St. Louis, MO 63178. The enzyme and albumin were dissolved in phosphate buffer to the desired concentrations. The relative molecular masses of HRP and albumin were assumed to be 44,000 and 66,120, respectively. Solutions of bilirubin and ethyl hydrogen peroxide (EtOOH; Accurate Chemical and Scientific Corp., Hicksville, NY 11801) were prepared as described previously (8).

Sera from umbilical cord blood as well as from 10 jaundiced, full-term, well infants (informed consent was obtained) were treated with ascorbate oxidase (EC 1.10.3.3, final concentration, 0.2 g/L) to reduce the interference from reduced ascorbate on the peroxidase reaction (9). The sera were adjusted to pH 7.4 with 0.30 mol/L KH\(_2\)PO\(_4\).

Potassium ferro- and ferricyanide were obtained from Mallinckrodt Laboratory Chemicals, St. Louis, MO 63147, and dissolved in distilled water to the desired concentration.

Methods

I used 2 \(\mu\text{mol/L}\) solutions of bilirubin to standardize the HRP solutions as described previously (8). I also used bilirubin concentrations of 0.05 and 0.10 \(\mu\text{mol/L}\), because the bilirubin concentrations ordinarily recommended for the standardization of HRP exceed the solubility of bilirubin at pH 7.4 (0.1 \(\mu\text{mol/L}\)), producing metastable solutions (10).

For the HRP standardization at low bilirubin concentrations, I used bilirubin dissolved in a small volume of 0.1 mol/L NaOH and diluted to a final concentration of 7.5 or 15.0 \(\mu\text{mol/L}\) with water containing 1 mmol of EDTA per liter. A 0.2-mL aliquot of this dilute bilirubin solution was then added to 29.8 mL of phosphate buffer at 37 °C. To determine the total bilirubin concentration from the absorbance at 440 nm, I used a Model 16 recording spectrophotometer (Cary Instruments, Monrovia, CA 96016) equipped with a cuvet of 10.0-cm pathlength and thermo-regulated at 37 °C. After adding HRP and EtOOH (500 \(\mu\text{mol/L}\)), I used the initial de-
crease in absorbance at 440 nm to calculate the rate constant (K) for the oxidation of bilirubin (8). Table 1 shows the results of the HRP standardizations. The mean rate constant, 643 L·μmol⁻¹·min⁻¹, was not significantly different at high and low bilirubin concentrations.

I calculated the AUBC in bilirubin-enriched sera or defatted albumin solutions (8) from the initial velocity (V) of the oxidation of bilirubin by HRP and EtOOH monitored at 37 °C and 460 nm, using a Model 240 recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074), and the equation:

$$\text{AUBC} = -V \cdot K \cdot (\text{HRP})^{-1}$$  (1)

where K is the rate constant (Table 1) and (HRP) is the concentration of horseradish peroxidase.

For total bilirubin concentrations >30 μmol/L, I used cuvets of 0.2- or 1.0-cm pathlength. Serum was diluted with phosphate buffer, or in some cases with serum ultrafiltrate obtained by filtering serum through CF 25 membrane cones (Amicon Corp., Lexington, MA 02173). In some experiments, I used potassium ferricyanide instead of HRP-EtOOH as the bilirubin oxidizing agent (11). The mean rate constant for the ferricyanide oxidation of bilirubin in non-albumin-containing solutions is 0.92 (SD 0.06) L·μmol⁻¹·min⁻¹.

To assess the effect of serum on HRP activity, I monitored the oxidation of potassium ferrocyanide (2 mmol/L) in 1.0 mL of phosphate buffer and various amounts of serum by HRP (0.11 μmol/L) and EtOOH (1.6 μmol/L) at 37 °C and 428 nm (Gilford 240). The absorptivities for potassium ferrocyanide and ferrocyanide at 428 nm were 90 L·mol⁻¹·cm⁻¹ and 950 L·mol⁻¹·cm⁻¹, respectively.

The dissociation rate constant (K₋₁) of the bilirubin–albumin complex was estimated from the ordinate intercept by using a modification of the equation derived by Faerch and Jacobsen (12):

$$B \cdot V^{-1} = a \cdot K_A \cdot K^{-1} \cdot (\text{HRP})^{-1} + (K₋₁)^{-1}$$  (2)

where B is the concentration of albumin-bound bilirubin (which is nearly equal to the total bilirubin concentration); V is the initial velocity of the oxidation of bilirubin by HRP and EtOOH; K_A is the equilibrium association constant for bilirubin and albumin; K is the rate constant for the oxidation of bilirubin by HRP and EtOOH, and a is the free albumin concentration, which can be estimated from the total bilirubin concentration minus the total albumin concentration when the bilirubin/albumin molar ratio is sufficiently low that second-site bilirubin binding is negligible. Linear plots were obtained by measuring either the change in velocity with change in HRP concentration, or the change in velocity with sample dilution, changing the total bilirubin and free albumin concentrations at a constant molar ratio for bilirubin/albumin and a constant HRP concentration.

**Results**

Determination of the AUBC at total bilirubin concentrations between 1 and 10 μmol/L with a constant bilirubin/albumin molar ratio (0.1) and HRP concentration (0.11 μmol/L) gave a linear decrease in the AUBC with dilution that was much more marked with umbilical cord serum than was true for defatted albumin solutions (Figure 1A). Further studies were undertaken to determine whether oxidation of albumin-bound bilirubin was contributing to the apparent decrease in unbound bilirubin concentration.

The total bilirubin concentration (C) at any sample dilution (but constant bilirubin/albumin molar ratio) is the sum of the concentrations of bound (B) and unbound (b) bilirubin. Dilution of solutions with a low molar ratio of bilirubin to albumin should affect C and B much more than b, and the total bilirubin at two dilutions differing by a factor X would be:

$$C_1 = B + b$$
$$C_2 = X C_1 = X B + b$$

If both the bound and unbound bilirubin are oxidized by HRP and EtOOH, the respective reaction velocities (V_1 and V_2) and apparent unbound bilirubin concentrations (AUBC_1 and AUBC_2) at C_1 and C_2 and a constant HRP concentration would be:

$$V_1 = K(b)(\text{HRP}) + M(B)(\text{HRP})$$
$$\text{AUBC}_1 = -V_1 \cdot K^{-1} \cdot (\text{HRP})^{-1}$$

$$V_2 = K(b)(\text{HRP}) + M(XB)(\text{HRP})$$
$$\text{AUBC}_2 = -V_2 \cdot K^{-1} \cdot (\text{HRP})^{-1}$$

where K is the rate constant in equation 1 (see Methods and Table 1) and M is the rate constant for oxidation of bound bilirubin by HRP and EtOOH. V_1 and V_2 (and thus AUBC_1 and AUBC_2) would not be equal, and results similar to those in Figure 1A would be expected.

If, however, the HRP concentration were also changed by a factor X at C_2 (i.e., the molar ratios of bilirubin/albumin and bilirubin/HRP are both constant at each dilution), then V_2 is increased by the factor X. AUBC_2, however, does not change, and results similar to those seen in Figure 1A would be expected.
\[
XV_2 = K(b)(XHRP) + M(X)(XHRP);
AUBC_2 = XV_2 \cdot K^{-1} \cdot (XHRP)^{-1} = -V_2 \cdot K^{-1} \cdot (HRP)^{-1}
\]

But repeating the dilution experiments in Figure 1A, keeping both the bilirubin/albumin and bilirubin/HRP molar ratios constant, did not give results similar to those in Figure 1A. Instead, the AUBC remained constant with sample dilution. Furthermore, it is apparent from the data in Figure 1B that the actual value for the AUBC depended entirely on the HRP/total bilirubin molar ratio selected for the analysis. This suggested that the HRP concentrations used in experiment 1A (0.11 \(\mu\)mol/L or 5.0 mg/L), although currently recommended for clinical use (1), may not be sufficiently rate-limiting as required for accurate determination of the unbound bilirubin concentration (8, 9). Furthermore, the dissociation rate constant for the bilirubin–albumin complex in serum would be lower than that for the complex in defatted albumin solutions, explaining the discrepancy between the two in Figure 1A. Extensions of dilution curves such as those in Figure 1A to higher total bilirubin concentrations should approach a maximum AUBC nearer to the actual unbound bilirubin concentration.

I extended the dilution curves of bilirubin-enriched sera or defatted albumin solutions to higher total bilirubin concentrations, keeping HRP concentration constant, and the curves indeed approached a maximum (Figure 2). Titration of the highest bilirubin concentrations with HRP (Figure 2, insets) resulted in loss of proportionality of the reaction velocity at much lower HRP concentrations for serum than for albumin. Calculation of \(K_{-1}\) with equation 2 in eight experiments similar to that in Figure 2 gave a mean dissociation rate constant for the bilirubin–albumin complex of 0.0033 (SD 0.0002) s\(^{-1}\) in serum, significantly (\(p < 0.001\)) lower than the value of 0.017 (SD 0.006) s\(^{-1}\) found with defatted albumin solutions.

If the dilution curves are totally the result of limited dissociation of the bilirubin–albumin complex, the AUBC should always be the same if the bilirubin/HRP molar ratio is kept constant at each dilution, even if the HRP concentration is excessive. The AUBC is equal to \(V \cdot K^{-1} \cdot (HRP)^{-1}\). Substituting AUBC \(K \cdot (HRP)\) for \(V\) as well as \(K_{1}/K_{-1}\) for \(K_{A}\) in equation 2 gives:

\[
AUBC = K_{-1}B \cdot [K_{A} + K(HRP)]^{-1}
\]

A dilution that decreases \(B\), \(a\), and HRP by the same factor should not affect the AUBC (i.e., the AUBC is the same before and after dilution). I determined the AUBC in defatted albumin solutions (409 \(\mu\)mol/L) containing bilirubin, before and after a 50-fold dilution. The bilirubin/HRP molar ratio was constant. The AUBC was nearly the same before and after dilution (Table 2), as predicted by equation 3.

I performed similar experiments on bilirubin-enriched cord serum and jaundiced infant sera, except that the “before-dilution” sample is actually a two-fold dilution of the stock serum with phosphate buffer, required to adjust the pH. The “after-dilution” sample is a 50-fold dilution of the stock serum. The results (Table 3) are quite different from the results with defatted albumin. The AUBC is 2.6- to 3.5-fold higher in the “before-dilution” sample, indicating that dilution-related factors other than the dissociation rate of the bilirubin–albumin complex affect the peroxidase test—possibly enhancement of HRP or bilirubin activity by serum, weak bilirubin binding competitors in serum, and (or) actual improvement of bilirubin binding with serum dilution.

The HRP activity was not enhanced by serum, as there was little change in the oxidation of ferrocyanide by HRP and EtOOH in the presence or absence of serum (Table 4). Undiluted serum slightly inhibited the reaction, probably through reconversion to ferrocyanide of the ferricyanide produced, by ascorbate and small amounts of other reducing agents in the serum. Furthermore, I obtained serum-dilution curves similar to those in Figure 2 on using ferricyanide instead of HRP.

### Table 2. AUBC before and after Dilution of Bilirubin–Defatted Albumin Solutions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bilirubin/albumin</th>
<th>HRP (nmol/L)</th>
<th>AUBC (nmol/L)</th>
<th>Ratio: AUBC before/AUBC after dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dilution</td>
<td>0.27</td>
<td>2.2</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>After dilution</td>
<td>0.27</td>
<td>2.2</td>
<td>3.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* 50-fold dilution.

### Table 3. Ratio of AUBC before/AUBC after Dilution in Cord and Infant Sera

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratio: AUBC before/AUBC after dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord</td>
<td>2.6 (±0.21)*</td>
</tr>
<tr>
<td>Infant</td>
<td>3.5 (±0.80)</td>
</tr>
</tbody>
</table>

* SD in parentheses.

### Table 4. Ferrocyanide Oxidation by HRP and EtOOH in the Presence and Absence of Serum

<table>
<thead>
<tr>
<th>Solution</th>
<th>Rate, (\mu)mol/min *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.66 (±0.05)</td>
</tr>
<tr>
<td>Serum/phosphate buffer, 1/1</td>
<td>1.05 (±0.07)</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>0.89 (±0.04)</td>
</tr>
</tbody>
</table>

* \(n = 3\), SD in parentheses.
EtOOH as the bilirubin oxidizing agent (Figure 3). I repeated the experiments summarized in Tables 2 and 3, using ferri-
cyanide instead of HRP and EtOOH, and found that the AUBC ratios before and after dilution were still 1.0 for de-
fatted albumin and 2.6 ± 0.2 for cord serum. The enhanced AUBC in undiluted serum does not appear to be unique to the HRP-EtOOH oxidizing system. No bilirubin binding competitors could be demonstrated, either by a decline in the AUBC in bilirubin-enriched undiluted cord serum after di-
alysis (10 mL of cord serum dialyzed against four 500-mL changes of phosphate buffer over 24 h) or by an increase in the AUBC in serum diluted with serum ultrafiltrate instead of phosphate buffer. The HRP concentration is constant (0.23 µmol/L) in these experiments, and the results are given in Table 5.

Thus, it would appear that either the oxidative activity of bilirubin is increased by serum or that dilution actually im-
proves the binding of bilirubin by serum.

**Discussion**

Although the peroxidase method is a very sensitive test for analysing bilirubin—albumin binding, it is clear that the re-
action conditions are extremely important and must be carefully controlled to obtain accurate data on concentrations of unbound bilirubin.

The data presented here indicate that serum behaves very dif-
ferently from solutions of defatted albumin, and suggest that the latter may have limited usefulness as a model for serum binding.

The reason(s) for the very slow dissociation of the biliru-
bin—albumin complex in serum is unclear. The phenomenon previously thought to be oxidation of albumin-bound bilirubin seen in partly purified albumin solutions, which disappears with defatting (9, 13), suggests that fatty acids may play a role in hindering the dissociation of the bilirubin—albumin complex. The influence of fatty acids on bilirubin transport and uptake is still unclear (14).

The improvement of bilirubin binding with serum dilution is suggested by the data presented by Cashore et al. (15), who noted differences in binding capacity when comparing sera analyzed by the standard peroxidase test (8) with a dialysis technique (MADDS method) in which undiluted serum is used (16). Furthermore, Lamola et al. (17), using a fluorometric test and undiluted whole blood, found primary bilirubin site binding constants 30 to 40 times lower than previously re-
ported, suggesting that peroxidase-determined estimates of the equilibrium association constant for the primary bilirubin binding site in serum may be high owing to excessive HRP concentrations, corrections for non-existent oxidation of bound bilirubin, and dilutional enhancement of binding.

Further work needs to be done to determine dilution-in-
fluenced factors in serum that affect bilirubin—albumin binding. Caution should be used in interpreting peroxidase determinations of the unbound bilirubin concentration if high sample dilutions or excessively high HRP concentrations are used, because the concentration of unbound bilirubin tends to be underestimated, giving a potentially false-negative result. Minimal dilution with low (<0.11 µmol/L) HRP con-
centrations may improve the accuracy of the unbound biliru-
bin concentration determination in serum. All the factors influencing serum binding of bilirubin must be evaluated, however, before the reliability of specific values for "un-
bound bilirubin" and risk for bilirubin toxicity can be as-
essed.

It is also apparent that factors other than serum bilirubin binding (e.g., acidosis) are extremely important in assessing risk for bilirubin toxicity in jaundiced infants (3). These fac-
tors will also need to be further evaluated before the appro-
riate relationships between serum bilirubin binding and bilirubin toxicity can be determined.

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**References**

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**Table 5. Effects of Dialysis and Dilution of Serum with Ultrafiltrate on AUBC in Cord Serum**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>AUBC, nmol/L</th>
<th>Before dialysis</th>
<th>After dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>95.7 (±4)*</td>
<td>97.8 (±5)</td>
</tr>
<tr>
<td>1:6</td>
<td>Phosphate buffer</td>
<td>33.3 (±2)</td>
<td>—</td>
</tr>
<tr>
<td>1:6</td>
<td>Serum ultrafiltrate</td>
<td>30.2 (±3)</td>
<td>—</td>
</tr>
</tbody>
</table>

*SD in parentheses; n = 3.*


