Bilirubin Binding by Primary Sites of Human Serum Albumin, with Suppression of Secondary-Site Interference by Use of a High Salt Concentration

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A high (2.0 mol/L) NaCl concentration apparently suppresses the secondary-site binding of bilirubin by human serum albumin. Thus if an excess of bilirubin is added to human serum albumin or to neonatal serum in buffer containing 0.1 mol of tris(hydroxymethyl)aminomethane and 2.0 mol of NaCl per liter (pH 7.5), and the bilirubin not bound to albumin is removed by treatment with calcium carbonate, the bilirubin binding reserve of primary binding sites can be estimated from direct measurement of spectral absorbance at 468 nm. Hemolysis and conjugated bilirubin apparently do not interfere. In a comparison study with serum samples from neonates the method gave results that generally agreed with those obtained by a commercially available Sephadex G-25 column procedure. In serum samples from adults the calculated unbound unconjugated bilirubin (free bilirubin) values derived by using binding reserves determined by the proposed method correlated well with the free bilirubin concentrations measured by a peroxidase method, but were only about one-half the amounts obtained by the peroxidase method.

Additional Keyphrases: neonatal chemistry • bilirubin binding reserve • suppression of binding by salt

I previously reported that human serum albumin had a molar bilirubin binding-capacity ratio of 3, according to a calcium carbonate adsorption technique (1). In this method, the adsorptive property of calcium carbonate was used to remove the bilirubin remaining unbound after addition of a supersaturating amount of bilirubin to albumin in tris(hydroxymethyl)aminomethane (Tris) buffer (0.1 mol/L at pH 7.5). More recently, it has been accepted that there are two types of binding sites on albumin: a single primary binding site of high affinity (2, 3) and at least two sites of lower affinity (2). At the secondary binding sites bilirubin can be readily displaced by water-soluble organic anions and is affected by pH variations (4, 5); bilirubin bound at the primary binding site, however, is not similarly affected (6). These differences have been ascribed to the fact that the primary binding site is hydrophobic and the secondary site is hydrophilic (7).

Decisions on whether to make exchange transfusions of hyperbilirubinemic neonates would be less difficult if there were a method for accurately estimating the reserve of unoccupied binding sites in the patient's serum. The limit of available primary binding sites is considered more important because bilirubin bound to weaker sites is assumed to be more dissociable and thus potentially manifested as bilirubin encephalopathies (8).

Of the methods reported to be of clinical value in estimating bilirubin binding reserve, those in which Sephadex G-25 is used to separate unbound bilirubin from bound bilirubin (9–11) are generally thought to provide the most nearly accurate estimate. Unfortunately, most of these methods are too cumbersome for clinical application, and the values they give are subject to variation because of changes in specimen or buffer volume, column flow speed, different batches of Sephadex, tightness of column packing, column diameter, etc. (12). Another disadvantage of the Sephadex methods is the apparent interference of conjugated bilirubin in concentrations exceeding 10 to 20 mg/L, which obviates use of these methods in neonates with hepatic excretory dysfunction.

In an in vitro study with bilirubin–albumin solutions in which mitochondria were used to sequester accessible bilirubin, either organic anions or hypertonic media caused an increased association of bilirubin with the mitochondria (6). As previously indicated, the organic anion effect is due primarily to competitive suppression of secondary-site binding of bilirubin. The effect of hypertonicity, however, had not been investigated further.

Thus, in this study, I chose a buffer containing 2.0 mol/L NaCl for investigation because of its effect in suppressing apparent binding of bilirubin sites. This effect is demonstrated in a comparative study involving spectrophotometric scanning, and in experiments that include the use of calcium carbonate for removal of the bilirubin not bound by albumin. On the basis of these observations, I propose a simple assay, which could be easily performed in most clinical laboratories, for estimating the bilirubin binding reserve of primary sites in human serum. The assay is compared with a commonly used commercial Sephadex method and a peroxidase method that measures the amount of unbound unconjugated bilirubin in human serum.

Materials and Methods

Instrumentation

All spectrophotometric measurements were made with the Beckman ACTA CIII spectrophotometer (Scientific Instrument Div., Beckman Instruments, Inc., Irvine, CA 92664). The band width was 0.2 nm at 468 nm, with instrument programming at other wavelengths. Semimicro 1.0-cm cuvettes were used for all measurements.

A rotating mixer (Model 150, Scientific Industries, Inc., Springfield, MA 01103) with the rotation speed set at 5 s per revolution was used in the basic procedure.

Reagents

Buffer grade Tris, 0.1 mol/L, was adjusted to pH 7.5; 0.1 mol/L Tris and 2.0 mol/L NaCl (Tris–salt buffer) was also adjusted to pH 7.5. Bilirubin solutions were prepared by dissolving bilirubin (Sigma Chemical Co., St. Louis, MO 63178) in cold 0.1 mol/L NaOH. The solutions were prepared in an
Proposed method. Cap and store polystyrene or acid-washed glass tubes that have been thoroughly rinsed and dried, 12 x 75 mm, containing 1.0 mL of appropriate buffer and 10 to 20 mg of CaCO₃. For the test, add 20 μL of the sample to one of the tubes. Use another tube, without sample, as the blank. Gently mix the contents of the tubes and store protected from light for 5 min; then centrifuge for 1 min at about 800 X g. Measure absorbance of the sample supernate at 468 nm, with the blank supernate as reference. Return the supernatants to their respective tubes and add to each 20 μL of freshly prepared bilirubin solution, adjusted to a concentration of 250 μg/L. Gently mix the contents of the tubes and store protected from light for 10 min, then cap the tubes and mix on a rotator, also protected from light, for 20 min. Centrifuge the tubes for 1 min at about 800 X g and again measure their absorbance at 468 nm. Calculate the binding reserve by multiplying the difference between the two absorbances by the previously derived absorptivity factor, F. If the binding reserve is <100 mg/L repeat the procedure, using 10 μL of the bilirubin solution (equivalent to a 125 mg/L bilirubin addition) instead of 20 μL.

Comparison methods. I used a clinically applicable method, Kernlute (Ames Company), with a slight modification. Add a 100-μL aliquot of the test serum to each of two columns. Add enough bilirubin to one column to adjust the bilirubin concentration in the serum aliquot to 5 mg/L less than the serum's binding reserve, as determined by the proposed method. To the second column, add the equivalent of 30 mg of bilirubin per liter more than the amount added to the first column. The rest of the procedure is as instructed by the manufacturer. I used the recently published method of Wennberg et al. (13) to measure unbound unconjugated bilirubin (free bilirubin). Other than performing it manually, I followed the procedure exactly.

Other methods. To measure total bilirubin values in sera from neonates, I used a Bilirubinometer (American Optical Corp., Buffalo, NY 14215). Conjugated bilirubin and albumin concentrations were determined with an acu (DuPont Instruments, Wilmington, DE 19898).

Results and Discussion

Calibration

The absorptivity factor (F) ranged from 449 to 459 (s = 4.1)
in 25 independent determinations on aliquots of Pediatric Versatol diluted with Tris–salt buffer. This is equivalent to a molar absorptivity of approximately 63 000.

**Spectrophotometric Scans**

Figure 1 shows scans of Tris buffer or Tris–salt containing 3.0 nmol of bilirubin before and after serial 2-μL additions of an albumin solution. It is apparent in Tris buffer that the spectral shift is almost complete after addition of 4.0 nmol of albumin, which strongly suggests that both primary and secondary binding sites are initially binding bilirubin. The isobestic point is poorly defined, suggesting a system consisting of mixed bilirubin–albumin complexes.

There are profound spectral changes in the presence of 2.0 mol/L NaCl in the buffer. The spectral shift is consistent with the occurrence of but one kind of binding site for bilirubin for each molecule of albumin. The use of salt concentrations at 1.0 and 1.5 mol/L gave similar but less reproducible scans than that illustrated. A salt concentration of 2.5 mol/L drastically diminished bilirubin binding, probably because of protein denaturation.

**Binding of Bilirubin by Albumin**

I assayed solutions containing 7.8 nmol of human serum albumin and different concentrations of bilirubin by the proposed method in two series of analyses, one with Tris buffer and the other with Tris–salt buffer as diluent. If only primary-site binding was occurring, one would expect that a maximum of 7.8 nmol of bilirubin would be bound and excess bilirubin would be removed by the calcium carbonate treatment. As seen in Figure 2, when Tris buffer was used as diluent, there was no plateau to indicate the occurrence of maximum binding at the primary site only; additional binding of bilirubin appeared to occur at secondary sites. With Tris–salt buffer as diluent, however, binding at secondary sites was suppressed, as demonstrated by a leveling off of bound bilirubin at approximately the primary-site saturation point of 7.8 nmol.

**Effect of Albumin Concentration in the Proposed Assay**

In preliminary studies with dilute human serum albumin solutions, some secondary site binding was apparent. For this reason, I added bilirubin equivalent to only 125 mg/L in assays involving less than 3.5 nmol of albumin; the 250 mg/L bilirubin solution was used for assays with more than 3.5 nmol of albumin. The data given in Figure 3 indicate the detection of nearly equimolar binding of bilirubin by albumin.

**Comparison of Methods**

Table 1 contains values obtained on serum samples from 15 premature neonates with conjugated bilirubin values of less than 15 mg/L. Albumin and unconjugated bilirubin concentrations were determined, and bilirubin-binding reserves were measured by the proposed method as well as by the modified Kernlute method. The binding reserves obtained by the Kernlute method were semi-quantitative and therefore are

<p>| Table 1. Bilirubin-Binding Reserve Estimate in 15 Serum Samples from Neonates |
|-----------------------------|-----------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>Albumin concn, mg/L</th>
<th>Unconjugated bilirubin, mg/L</th>
<th>Proposed method</th>
<th>Kernlute method (range)</th>
<th>Bilirubin-binding capacity, mg/L.a</th>
</tr>
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<tbody>
<tr>
<td>21.0</td>
<td>81</td>
<td>54</td>
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<td>36.3</td>
<td>128</td>
<td>121</td>
<td>116–146</td>
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</table>

a Bilirubin-binding reserve obtained by proposed method, added to the serum concentration of unconjugated bilirubin. b Nonagreement of proposed method with Kernlute method.
reported as a range in which the lower value indicates negative
detectable unbound bilirubin and the higher value indicates
a positive response. With 12 of the samples there was a general
agreement between the two methods.

Sixteen serum samples from adults, with conjugated bilirubin
values below 5 mg/L and different amounts of unconjugated bilirubin,
were selected for the analysis by the proposed method and by a
peroxidase method for free bilirubin. The binding reserve obtained by the proposed method, the
unconjugated bilirubin concentration (from total and conjugated assays), and the published affinity constant (14) of
the primary site of albumin at room temperature, \(2.0 \times 10^8\), were
used to calculate the free bilirubin in the samples. Correlation of the calculated values with the peroxidase method values
was good, as seen in Figure 4. The calculated values, however,
are about 50% of the measured values. In part, this low proportionality may be explained by the fact that the peroxidase
method does not correct for any oxidation of bound bilirubin;
alternatively, the affinity of the primary binding site of albumin for bilirubin may be enhanced by the high salt concentration
used in the proposed method.

Effect of Hemolysis and Conjugated Bilirubin

Ten visibly hemolyzed sera were used as samples, subjected
only to the calcium carbonate treatment used in the proposed
method with Tris–salt buffer as diluent. There was no differ-
ence in the absorbance at 468 nm before and after the treatment.
Ten serum samples with increased conjugated bilirubin concentrations (51 to 183 mg/L) were similarly
subjected to the calcium carbonate treatment. The decrease in absorbance, due to adsorption or destruction of conjugated
bilirubin, was less than 4.5 mg/L (\(\Delta A <0.01\)) for all 10 sera.
Thus the contributions of hemoglobin and conjugated bilirubin to absorbance at 468 nm were unaffected by the calcium carbonate treatment used in the proposed method.

The experimental evidence presented suggests that high
salt concentrations suppress the secondary-salt binding of
bilirubin by human serum albumin. With secondary sites
apparently suppressed, bilirubin added to albumin or serum
is bound only by primary sites. Surplus bilirubin remains un-
bound and is removable by a calcium carbonate treatment.
The method I propose for the estimation of primary site
binding reserve is not only simple to perform, it also is una-
fected by high amounts of conjugated bilirubin. This advan-
tage enables evaluation of bilirubin binding reserve in neo-

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