Improved Method for Accurate Quantitation of Total and Conjugated Bilirubin in Serum

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We describe an improved method for the quantitation of total and direct bilirubin in serum in which sodium dodecyl sulfate is used as an accelerator. Viscous and caustic reagents are not needed, and the effects of protein matrix, hemoglobin interference, and turbidity are minimized. For total bilirubin determination we first add the sodium dodecyl sulfate reagent to serum, then a diazo reagent that lowers the pH to 5.6, the effective pKₐ of maleic acid. After 10 min we add sulfanilic-HCl reagent to bring the pH to 1.55. For determination of conjugated bilirubin, we add the sulfanilic-HCl reagent first, followed by the diazo reagent, an ascorbic acid solution, and finally, the sodium dodecyl sulfate reagent. The same sample blank is used for determination of both total and direct bilirubin. At 565 nm the molar absorptivity of azobilirubin determined with Standard Reference Material 916 in human serum albumin, bovine serum albumin, and pooled serum is 82780 ± 496 L·mol⁻¹·cm⁻¹. The absorptivity remains constant to within 0.3% in the pH range of 1.45 to 1.67. The color at both pH 5.6 and 1.55 is stable to within 0.1% for at least 1 h. The method is unaffected by protein concentrations of 2–120 g/L, has a linear standard curve to within 0.5% for bilirubin concentrations of 0–250 mg/L, and correlates well with the Jendrassik–Grof method by linear-regression analysis of 52 samples from patients (r = 0.999; slope, 1.02; y-intercept, 0.27 mg/L). We discuss a mechanism to explain the effects of hemolysis on the coupling of the diazo reagent, and discuss the effects of several hemebinding agents that can be used to minimize such interference.

Additional Keyphrases: variation, source of · spectrophotometry · mechanism(s) of bilirubin release from albumin

The laboratory determination of bilirubin is most commonly performed by the Malloy–Evelyn (1, 2), the Meites–Hogg (3), or the Jendrassik–Grof (4, 5) procedures. The Malloy–Evelyn and Meites–Hogg methods suffer from high blank absorbances, which result from sample turbidity, and a marked dependence on both the composition of the protein matrix and the concentration of endogenous hemoglobin. The Jendrassik–Grof method, which suffers less from sample turbidity and hemoglobin effects than the other methods, requires the use of caustic and viscous reagents. We report here the details of our method, which uses the detergent sodium dodecyl sulfate (SDS) to overcome the above difficulties in the determination of total and conjugated bilirubin. The use of detergents for bilirubin analysis has been reported by Perlman and Lee (6), who used Duponol, and by Watanabe et al. (7), who used SDS. Inadequate attention to considerations of standardization, protein concentration, matrix effects, and pH optimization leads to low sensitivity and questionable accuracy in these methods. In developing our method we have relied extensively on the excellent approach of Douglas et al. (8) in their evaluation of the Malloy–Evelyn, the Meites–Hogg, and the Jendrassik–Grof methods.

Material and Methods

Apparatus

Absorption measurements were performed on a Cary Model 118C spectrophotometer (Varian Instruments, Monrovia, CA 91016) with the gain adjusted so that the slit widths were controlled between 0.01 and 0.03 mm (spectral bandwidth, 0.6–1.1 nm). We used a Model 300-N spectrophotometer (Gilford Instruments, Oberlin, OH 44074) with a 10-mm pathlength cuvette only for the correlation studies between our method and the Jendrassik–Grof method. The wavelength accuracy and linearity were checked with Standard Reference Material (SRM) 931 liquid filters for spectrophotometry (National Bureau of Standards, Washington, DC 20234). We used SRM 136-C potassium dichromate and cobalt ammonium sulfate (Matheson, Coleman and Bell, Norwood, OH 45212) as further checks on both spectrophotometers. A “Suprasil (QS)” 10 ± 0.1 mm cuvette (Helma Cells, Inc., Jamaica, NY 11424), calibrated against a SRM 932 quartz cuvette, was used for all measurements in both spectrophotometers.

We used class A volumetric glassware throughout except in the hemoglobin studies and the correlation studies with Jendrassik–Grof, where Eppendorf pipettes and Brinkmann dispensers were used (Brinkmann Instruments, Westbury, NY 11590). The Eppendorf pipettes and Brinkmann dispensers were calibrated by weighing the water delivered. All glassware was washed in acid and thoroughly rinsed with Type I (9) de-ionized water.

We measured pH values with a Model 601A/Digital ionalyzer (Orion Research, Cambridge, MA 02139). Millipore filters HAWP (0.45 μm, 25 mm), prefilters AP15 (25 mm), and Swinnex filters (25 mm) were purchased from Millipore Corp., Bedford, MA 01730.

We used a Model H51 balance (Mettler Instrument Co., Princeton, NJ 08540), which we calibrated with Class S weights.

Chemicals

Bilirubin: SRM 916 and bilirubin from Fisher Scientific Co., Silver Spring, MD 20910, were both used without further
purification. The SRM 916 bilirubin was used only for the determination of molar absorbptivities.

**Bovine serum albumin:** No. 12589; Calbiochem, San Diego, CA 92122.

**Human serum albumin:** No. A-2386; Sigma Chemical Co., St. Louis, MO 63178.

**Pooled human serum:** We prepared pooled serum in our laboratory in accordance with the specifications of The Joint Committee Report (10).

**Control serum:** Fisher control serum (Fisher) and PACP I and PACP II (Laboratory Supply Co., Pittsburgh, PA 15230).

**Sodium dodecyl sulfate (SDS):** Sigma; or sequanual grade, from Pierce Chemical Co., Rockford, IL 61051.

**Maleic acid diosodium salt** (purim grade): Tridom/Fluka, Hauppauge, NY 11787.

**Maleic acid:** Eastman Kodak Co., Rochester, NY 14650, and Mallinckrodt Inc., St. Louis, MO 63147.

**Ascorbic acid, caffeine, and pyridine:** Eastman Kodak.

**Dimethyl sulfoxide, ethylenediaminetetraacetic acid,** hydrochloric acid, sodium carbonate, sodium benzoate, sodium hydroxide, sodium nitrite, sodium tartrate, sucinic acid and, 1,10-phenanthroline (monohydrate): Fisher.

**Sodium acetate, sodium chloride, and sufanilic acid:** J. T. Baker Chemical Co., Phillipsburg, NJ 08865.

**8-Hydroxyquinoline and nicotinamide:** Sigma.

**Nicotine:** Courtesy of Dr. Yale Caplan.

**NAD:** LDH-L SuperSTAT Pack, Calbiochem.

**NADH:** GOT 500 Pack, Calbiochem.

**Histidine:** ICN Pharmaceuticals, Inc., Cleveland, OH 44128.

**Bilirubin Standards**

Prepare stock solutions of bovine or human serum albumin, 60 g/L, in saline, 9 g/L, and adjust the pH to 7.4 with sodium hydroxide. Weigh bilirubin to the nearest 0.05 mg. Prepare bilirubin standards in a darkened room according to the method described by Doumas et al. (8). Disperse bilirubin with 1 mL of dimethyl sulfoxide in a 20-mL Pyrex beaker, then add 2 mL of sodium carbonate, 0.1 mol/L, which will completely dissolve the bilirubin after several minutes. After adding bovine or human albumin solution to the beaker, transfer the contents to a volumetric flask via a polypropylene funnel. Repeatedly rinse the beaker with fresh protein solution, and when the flask is 80% full, add approximately 2 mL of HC1 (0.1 mol/L) to neutralize the sodium carbonate. The exact volume of HC1 is determined by titration with the sodium carbonate to a phenolphthalein end point. Bring the solution to its final volume with protein stock solution. Use freshly prepared standards for all experiments.

**Reagents for the SDS Method**

**Sodium dodecyl sulfate reagent.** Add 20 g of maleic acid diosodium salt (0.125 mol) to 500 mL of de-ionized water while stirring at approximately 50 °C. Follow with 4 g of caffeine and 50 g of SDS, making certain that each reagent is dissolved before adding the next one. Dilute the solution to approximately 950 mL, cool to room temperature, and adjust to pH 8.2 with sodium hydroxide or hydrochloric acid, 1 mol/L. To prevent a drift in the pH readings, the electrode must be equilibrated with the SDS solution for 10 min before calibration of the pH meter. Gently transfer the solution to a volumetric flask (avoid foaming) and add sufficient de-ionized water to bring the final volume to 1 L. Filter this solution and store in polypropylene bottles at room temperature.

In an alternative but less desirable procedure, 14.5 g of maleic acid and 9.6 g of sodium hydroxide can replace the maleic acid diosodium salt. It is critical to avoid an excess of sodium hydroxide to prevent excessive foaming.

Although SDS is known to hydrolyze at low pH, we have found the reagent to be stable for this assay for at least six months.

**Sulfanilic acid reagent.** Add 6.7 g of sulfanilic acid to 800 mL of water in a beaker with stirring. When the sulfanilic acid is completely dissolved, add 20 mL of concentrated HCl, transfer to a 1-L volumetric flask, and bring to 1 L with water.

**Ascorbic acid reagent.** Add 0.1 g of ascorbic acid per milliliter of sulfanilic acid as needed each day.

**Sodium nitrite reagent.** Add 0.5 g of sodium nitrate to 100 mL of water. This solution is stable at 4 °C for at least one week.

**Diazo reagent.** Mix 0.3 mL of sodium nitrite reagent with 9 mL of sulfanilic acid reagent. Use within 1 h after preparation.

**Saline reagent.** Dissolve 9 g of sodium chloride per liter of de-ionized water.

**Reagents for Jendrassik–Grof Method**

All reagents were made up as described by Doumas et al. (8) except for the ascorbic acid reagent, which we prepared by dissolving 0.2 g of ascorbic acid in 5.0 mL of de-ionized water. Prepare fresh reagents daily.

**SDS Procedure**

**Total bilirubin.** To 0.1 mL of serum add 1.0 mL of the SDS reagent and mix well (we recommend vortex-mixing). Add 0.3 mL of the diazo reagent, mix well, wait 10 min, then add 1.0 mL of the sulfanilic acid reagent. Prepare the blank exactly the same way but replace the diazo reagent with 0.3 mL of ascorbic acid reagent. Read absorbance at 565 nm against water. The ascorbic acid prevents oxidation of bilirubin in the blank, and does not absorb at 565 nm.

**Conjugated bilirubin.** To 0.1 mL of serum add 0.5 mL of the sulfanilic acid reagent and mix. Add 0.5 mL of the diazo reagent and mix. After 1 min, add 0.3 mL of ascorbic acid reagent, then 1.0 mL of the SDS reagent; mix thoroughly. Use the same blank as in the procedure for the total bilirubin. Read absorbance at 565 nm. When the SDS is added, a slight turbidity may occur that will disappear immediately upon mixing (see Results and Discussion).

**Jendrassik–Grof Procedure**

**Total bilirubin.** To 0.1 mL of serum add 1.0 mL of the caffeinnamon and mix well. Ten minutes after adding 0.4 mL of the diazo reagent, add 0.6 mL of the alkaline tartrate reagent. Prepare the blank exactly the same way but replace the diazo reagent with 0.4 mL of the sulfanilic acid reagent. Read absorbance at 600 nm against water.

**Conjugated bilirubin.** This method is a modification of that described by Gambino (5). To 0.1 mL of serum add 0.95 mL of HCl (0.05 mol/L); mix and add 0.4 mL of the diazo reagent. After 1 min, add 0.05 mL of ascorbic acid reagent, then add 0.6 mL of alkaline tartrate. For the blank, use 0.4 mL of sulfanilic acid in place of diazo reagent. Mix thoroughly and read absorbance at 600 nm against water.

**Results and Discussion**

**Experimental Considerations**

**Maleic acid buffering capacity.** To obtain high buffering capacity at the pH of diazo coupling (about 5.6) and at the final pH (about 1.6), we examined the buffering capacity of a number of phosphate and carboxylic acid systems. With phosphate buffers, the color produced was unstable at the pH of diazo coupling. Succinic acid has pH's near the desired pH value; however, high concentrations (at least 0.25–0.50 mol/L)
were required for adequate buffering capacity, and such high concentrations were considered undesirable. The best buffer system we found was maleic acid.

We prepared stock solutions (50 g/L) of SDS in 0.1, 0.125, and 0.15 mol of maleic acid per liter and added 1.0 mL of a 200 mg/L standard of bilirubin in bovine serum albumin (BSA) to 10 mL of each of these solutions. We then titrated the solutions with the sulfanilic acid reagent to simulate the diazo reaction. The results are shown in Figure 1. It is evident that for the 0.125 mol/L concentration, which is used in the SDS method, there is considerable buffering capacity at both the stage of diazo coupling and the stage at which the absorbance is determined. The coupling occurs at a pH equal to the effective pK₂ of 5.6, and the absorbance is read at a pH of 1.55, which is the effective pK₁. Additional buffering at pH 1.55 is contributed by the HCl (0.24 mol/L) in the sulfanilic acid reagent. Even a 10% error in the total amount of sulfanilic acid reagent added will result in an insignificant pH error of only 0.05 at pH 1.55.

**Absorbance vs pH.** We prepared 225 mL of azobilirubin from a 200 mg/L standard of BIL-BSA in the proportions described in the SDS procedure. After pipetting 15-mL aliquots into fourteen 20 × 150 mm screw-top test tubes, we added HCl to each of the azobilirubin-containing test tubes to produce a series with 14 different pH values. The absorbance and pH of each solution were determined immediately (Table 1). The final pH at which the absorbance is read in test samples ought to be between 1.50 and 1.60 because of the strong buffering capacity of the maleic and hydrochloric acids. Note from Table 1 that the absorbance range of ±0.3% at pH 1.50–1.67 is well within the range of uncertainty to be expected from random errors in the determination of pH and absorbance.

**Optimization of SDS concentration.** We added 10 mL of SDS reagent (range 10–80 g of SDS per liter) to 1 mL of a BIL-BSA standard (200 mg/L), then added 3 mL of diazo reagent. After 10 min, 10 mL of sulfanilic acid reagent was added and the absorbance determined immediately. As shown in Table 2, at least 30 g of SDS per liter must be added to completely release the bilirubin from the protein; moreover, there is no change in absorbance from 30 to 80 g of SDS per liter. The chosen optimum concentration of 50 g/L results in 20.8 g of SDS per liter in the final reaction mixture.

**Table 1. Effect of pH on Absorbance of Azobilirubin at 565 nm**

<table>
<thead>
<tr>
<th>pH</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.33</td>
<td>0.515</td>
</tr>
<tr>
<td>2.77</td>
<td>0.855</td>
</tr>
<tr>
<td>1.87</td>
<td>1.028</td>
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<tr>
<td>1.84</td>
<td>1.032</td>
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<td>1.82</td>
<td>1.036</td>
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<td>1.81</td>
<td>1.035</td>
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<td>1.72</td>
<td>1.040</td>
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<tr>
<td>1.71</td>
<td>1.040</td>
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<tr>
<td>1.67</td>
<td>1.044</td>
</tr>
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<tr>
<td>1.50</td>
<td>1.045</td>
</tr>
<tr>
<td>1.44</td>
<td>1.050</td>
</tr>
<tr>
<td>1.25</td>
<td>1.058</td>
</tr>
</tbody>
</table>

* The bracketed portion emphasizes that absorbance in this pH range is 1.045 ± 0.003, which is an uncertainty of 0.3%.

**Optimization of sulfanilic acid concentration.** We diluted the stock sulfanilic acid reagent (6.7 g/L) to 1.0, 2.5, and 5.0 g/L with a HCl solution of 20 mL of concentrated HCl per liter. We prepared four diazo reagents by adding 0.3 mL of sodium nitrite (5 g/L) to each of the sulfanilic acid reagents. It is evident, from the constant absorbance produced with a 200 mg/L standard of BIL-BSA (Table 2), that the method is insensitive to sulfanilic acid concentrations between 2.5 and 6.7 g/L. We used a concentration of 6.7 g/L, to give the same total sulfanilic acid concentration in the SDS reaction mixture as in the Jendrassik–Grof method.

**Optimization of sodium nitrite.** We added various amounts of the 5 g/L stock sodium nitrite reagent to 9-mL volumes of the 6.7 g/L sulfanilic acid reagent, which were subsequently used as diazo reagents. The results of the absorption measurements on adding the diazo reagents to a 200 mg/L standard of BIL-BSA are shown in Table 2. The measured absorbances demonstrate that at least 0.1 mL of sodium nitrite reagent is required; however, the method is not critically sensitive to the amount of added sodium nitrite over the range 0.1–0.5 mL. We chose to use 0.3 mL, to provide the same final concentration of sodium nitrite as that used in the Jendrassik–Grof method.

**Rate of reaction and color stability.** We mixed 0.2 mL of a 200 g/L standard of BIL-BSA with 2 mL of SDS in a 1-cm cuvette, then added 0.6 mL of diazo reagent. The contents

**Table 2. Azobilirubin Absorbances at 565 nm as a Function of SDS, Sulfanilic Acid, and Sodium Nitrite Concentrations**

<table>
<thead>
<tr>
<th>SDS</th>
<th>Sulfanilic Acid</th>
<th>NaNO₂ reagent (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/L</td>
<td>g/L</td>
<td>mL</td>
</tr>
<tr>
<td>10</td>
<td>1.040</td>
<td>1.0 1.188</td>
</tr>
<tr>
<td>20</td>
<td>1.088</td>
<td>2.5 1.212</td>
</tr>
<tr>
<td>30</td>
<td>1.090</td>
<td>5.0 1.212</td>
</tr>
<tr>
<td>40</td>
<td>1.094</td>
<td>6.7 1.212</td>
</tr>
<tr>
<td>50</td>
<td>1.091</td>
<td>1.212</td>
</tr>
<tr>
<td>60</td>
<td>1.095</td>
<td>1.212</td>
</tr>
<tr>
<td>80</td>
<td>1.093</td>
<td>1.212</td>
</tr>
</tbody>
</table>

* Different preparations of bilirubin (200 mg/L) in bovine serum albumin were used for studies with each reagent.

* Concentrations chosen as optimum.

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were mixed with a Pasteur pipette, and the change in absorbance with time was recorded at 530 nm, the wavelength of absorption maximum of azobilirubin at pH 5.6. The reaction at pH 5.6 is complete within 2 min, and the absorbance is stable to within 0.1% for at least 1 h. The absorbance at the final pH of 1.55 is also stable to within 0.1% for at least 1 h.

Effect of protein. From a 50-mL stock solution of bilirubin in 40 g of bovine serum albumin per liter, we transferred 6 mL into each of seven 25-mL volumetric flasks. The flasks were brought to final volume with various proportions of a 15 g/L solution of bovine serum albumin (in saline, pH 7.4) and saline to produce the desired range of protein concentrations (10–120 g/L). The bilirubin content of each sample was determined in duplicate, and the average absorbance for all protein concentrations was 0.829 ± 0.003 (SD), with a coefficient of variation (CV) of 0.36%. Thus for a range in serum protein concentrations of 10–120 g/L, the absorbance uncertainty was only three parts in 829, which is negligible in a routine bilirubin determination.

Linearity

Aliquots from stock solutions of approximately 250 mg of bilirubin per liter in 60 g/L solutions of bovine serum albumin or pooled human serum were pipetted into 25-mL volumetric flasks to produce final concentrations of 5, 10, 50, 100, 150, 200, and 250 mg/L when diluted with bovine serum albumin, pooled serum, or saline. All measurements were performed in duplicate and the results are shown in Table 3. When saline was used as the diluent, the protein concentration ranged from 1.2 to 60 g/L and, consequently, we could assess simultaneously both the linearity of the standard curve of the method and the effect of protein concentration. For a given protein matrix, the curve for concentration vs absorbance with the SDS method is linear from 0 to 250 mg of bilirubin per liter; this linearity is not affected by variable protein concentrations in the range of 1.2–60 g/L for bovine serum albumin or pooled serum (see above, Effect of protein).

Molar Absorptivity

We weighed 10 mg of SRM bilirubin to ±0.05 mg, and prepared standards in bovine or human serum albumin or pooled human serum in 50-mL volumetric flasks. The molar absorptivities were determined immediately. We made three separate preparations for each protein matrix and determined the molar absorptivities five times for each preparation. Calculations of the molar absorptivity were as described by Doumas et al. (8). The mean value for all 45 determinations considered together is 82,780 ± 496 L-mol⁻¹-cm⁻¹ (CV 0.6%).

Table 3. Linearity of the SDS Method

<table>
<thead>
<tr>
<th>x</th>
<th>y</th>
<th>y’</th>
<th>x</th>
<th>y</th>
<th>y’</th>
<th>x</th>
<th>y</th>
<th>y’</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.028</td>
<td>0.023</td>
<td>10</td>
<td>0.054</td>
<td>0.052</td>
<td>50</td>
<td>0.280</td>
<td>0.283</td>
</tr>
<tr>
<td>100</td>
<td>0.569</td>
<td>0.572</td>
<td>150</td>
<td>0.856</td>
<td>0.861</td>
<td>200</td>
<td>1.148</td>
<td>1.151</td>
</tr>
<tr>
<td>250</td>
<td>1.447</td>
<td>1.440</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

m = 0.00578 | m = 0.00572 | m = 0.00570
b = −0.00606 | b = −0.00440 | b = −0.00216
S_m = 0.0000227 | S_m = 0.00000653 | S_m = 0.0000122

x, concentration of bilirubin in mg/L; y, observed absorbance; y’, absorbance calculated from least-squares analysis y’ = mx + b; m, slope of the least-squares line; b, intercept of the least-squares line; S_m, standard deviation of the slope.

* Bilirubin dissolved in bovine serum albumin (BSA) or pooled serum (PS) and diluted with albumin, serum, or saline (see text for details). Values are averages of duplicate determinations.

Fig. 2. Linear regression analysis of the SDS and the Jendrassik–Grof methods.
coefficient is 0.998. Thus, over the range of 0–200 mg of bilirubin per liter, the SDS and Jendrassik–Grof methods show excellent agreement. An apparent slight positive bias of the SDS method at concentrations between 100 and 200 mg/L is insignificant and is probably related to the uncertainty in the azobilirubin molar absorbivities of the two methods.

**Effect of Hemoglobin**

A 100 g/L stock solution of hemoglobin (Hb) was prepared from washed erythrocytes by freezing and thawing in water. Concentrations were determined by a spectrophotometric method (11). Necessary dilutions were made with de-ionized water. Standard solutions containing Hb and bilirubin in pooled serum were made in 25-mL volumetric flasks to final Hb concentrations of 1.0, 2.5, 5.0, and 10.0 g/L. We studied analytical recovery by measuring the amounts of bilirubin in samples with and without added Hb.

The results of the recovery studies for the SDS method with and without caffeine and for the Jendrassik–Grof method are shown in Table 4. The recoveries for the SDS method with caffeine and for the Jendrassik–Grof method are comparable; however, in the absence of caffeine, the recovery for the SDS method is quite unsatisfactory at high Hb concentrations.

We did not use caffeine in our initial studies on the SDS method, but when we discovered that the Hb effect was so large, we investigated the nature of the Hb interference.

To determine whether the lower recoveries in the SDS method were a result of an azobilirubin–hemoglobin complex that lowered the molar absorbivity or shifted the wavelength of maximum absorbance, we added 0.1 mL of a 10 g/L solution of either bovine serum albumin or Hb to 2.5 mL of an azobilirubin solution freshly prepared from a BIL-BSA standard (200 mg/L). Because the maximum absorbance (corrected for blank absorbance) of the two samples occurred at 565 nm and differed by only 0.3%, we concluded that there was neither a wavelength shift nor a lowering of the molar absorbity by an azobilirubin–hemoglobin complex, if such a complex exists. Therefore, the possibility that Hb interferes with the diazo coupling must be considered.

We determined the exact stage in the reaction procedure at which hemoglobin interferes by measuring the absorbance of samples in which the sequence of addition of the reagents and Hb was varied. Only when Hb addition preceded diazotization was Hb interference evident, as judged by the decreased recoveries (Table 5, experiments 4 and 5). Thus we conclude that Hb interferes with the diazo reaction.

Kinetic studies have shown that in the presence of sufficient diazonium salt the formation of azobilirubin occurs in at least two steps (12, 13): the formation of azobilirubin by cleavage of the central methylene bond (Figure 3) and the coupling of the diazonium salt with hydroxypyrromethene carbinol to form a second molecule of azobilirubin. The similarity of the pyrrole moieties of bilirubin and of hydroxypyrromethene carbinol to the structures of compounds known to bind heme (14) is suggestive of a possible heme inhibition through the formation of a heme–bilirubin or a heme–hydroxpyrromethene carbinol complex. An example of such a complex between the pyrrole moiety of hydroxypyrromethene carbinol and heme is depicted schematically in Figure 3 and, as shown, would inhibit formation of the second molecule of azobilirubin. We emphasize that we have no direct evidence for the existence of either complex described above; however, our experimental observation that recoveries are inversely proportional to the concentration of Hb and directly proportional to the concentration of bilirubin (Table 4) is consistent with the existence of either one or both of these complexes.

To ascertain if added heme-binding compounds could eliminate heme interference by competitively binding heme,
we made analytical recovery studies. To 0.1 mL of de-ionized water or 0.1 mL of hemoglobin stock solution (100 g/L) we added 0.1 mL of bilirubin (100 mg/L) in bovine serum albumin. We then added 0.1 mL of the heme-binding compound, diazotized the samples as described in Materials and Methods, and determined the absorbances at 565 nm. The results, shown in Table 6, demonstrate that, although the effect of Hb interference could not be entirely eliminated at high concentrations, recovery could be substantially increased with the addition of heme-binding compounds. We have chosen to use caffeine in our method because it is inexpensive, readily available, safe, and easy to handle. The caffeine used as an accelerator in the Jendrassik–Grof method undoubtedly aids in reducing Hb interference.

**Conjugated Bilirubin**

Because primary standard bilirubin glucuronides are not available, nothing is known about the absolute accuracy of any method for measuring conjugated bilirubin. We have observed widely divergent results with different methods in current use. However, with a given method, relative estimates of conjugated bilirubin can provide clinically useful information. We have therefore used the reagents optimized for total bilirubin analysis to develop a method for conjugated bilirubin by adjusting reagent volumes to obtain results identical to those of the Jendrassik–Grof method described in Material and Methods. Our method for conjugated bilirubin thus compares well with a method that has experienced widespread clinical usage (5). Beyond stating that our method for conjugated bilirubin produces clinically useful results, we make no statements about the accuracy of this or any method for conjugated bilirubin.

**Action of SDS**

Although we have not performed experiments to elucidate the details by which SDS promotes the release of bilirubin

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**Table 6. Analytical Recovery of Bilirubin after Addition of Heme-Binding Compounds**

<table>
<thead>
<tr>
<th>Compound added and concn, mol/L</th>
<th>Bilirubin recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>76</td>
</tr>
<tr>
<td>1,10-Phenanthroline, satd.</td>
<td>87</td>
</tr>
<tr>
<td>Bathophenanthroline, satd.</td>
<td>85</td>
</tr>
<tr>
<td>8-Hydroxyquinoline, satd.</td>
<td>86</td>
</tr>
<tr>
<td>Histidine, 0.01</td>
<td>85</td>
</tr>
<tr>
<td>Pyridine, 0.08</td>
<td>88</td>
</tr>
<tr>
<td>NAD+, 0.01</td>
<td>91</td>
</tr>
<tr>
<td>Nicotine, 0.01</td>
<td>92</td>
</tr>
<tr>
<td>Nicotinamide, 0.01</td>
<td>88</td>
</tr>
<tr>
<td>Caffeine, 0.02</td>
<td>89</td>
</tr>
</tbody>
</table>
from serum albumin, we feel that it is instructive to point out some of the possible modes of action based upon and consistent with published observations of the interaction between SDS and serum albumin.

The interaction between detergents and proteins has been the subject of laboratory investigation for over four decades and has been extensively reviewed (15–18). SDS has been shown to bind to serum albumin through a combined ionic and hydrophobic interaction (19–20). Charged groups of serum albumin interact with the ionic head group of SDS, and hydrophobic regions interact with the hydrocarbon tail of the detergent molecule. There are about 10–12 high-affinity binding sites for SDS in serum albumin (18, 21) and, although they are usually saturated at low detergent concentrations, this binding has a minimal effect on protein conformation. Binding to additional sites of lower affinity is thought to result from exposure of weaker binding sites in a cooperative alteration of the native structure of the protein; when more than about 20 molecules of SDS have been bound, the protein undergoes a series of denatured states.

In the SDS method we describe, the concentration of SDS during the diazotization of bilirubin is 0.124 mol/L (total volume 1.4 mL) and the approximate molar ratio of SDS to protein ranges from 1000:1 (10 g/L protein) to 100:1 (120 g/L protein). At the ionic strength used in this test the critical micelle concentration is on the order of 1 mmol/L (19, 22, 23). Because the total molar concentration of SDS of 0.124 mol/L exceeds the critical micelle concentration by more than 100-fold, 99% of all the SDS is present in the form of micelles. The micelles have approximately 100 SDS molecules per micelle (24, 25) and, therefore, the molar concentration of SDS micelles in this system is approximately 1 mmol/L.

However, the important parameter in the assessment of SDS binding to protein is the free SDS monomer concentration and not the total concentration of detergent or the micellar concentration. Furthermore, monomer concentrations >0.1 mmol/L are sufficient to disrupt the native conformation of the protein (18, 26). Because the concentration of free monomer in the SDS test is approximately 1 mmol/L, perhaps one mode by which SDS promotes the release of bilirubin is through a disruption of the conformation of the bilirubin-binding site, with a concomitant release of bilirubin into solution.

Accelerators for bilirubin analysis have included acetate, benzoate, methyl or ethyl alcohol, pyridine, urea, caffeine, salicylate, and diphyllyl (27). These compounds have been used alone and in some combination with each other. Lolekha and Limpathayakul (28) have shown that acetate, urea, or caffeine used alone, unless in very high concentrations, will not promote the complete release of bilirubin. However, when these reagents are combined, release is complete at much lower concentrations of each reagent, which strongly suggests that an effective accelerator must be capable of both ionic and hydrophobic interactions; SDS, by virtue of its amphiphilic nature, meets these requirements.

Once bilirubin is released from serum albumin, the bilirubin will almost certainly partition between the micelles and the solvent. SDS can release heme from hemoglobin and this free heme as well as the caffeine can partition into the micelles along with serum components such as cholesterol (29). Porphyrins are known to partition into SDS micelles (30, 31) and, in particular, the partition coefficient for heme has been determined (32). The hemin is thought to be located radially in the micelle with the carboxyl groups facing outward toward the solvent (32, 39) and with the iron also available to the aqueous environment.

It is possible that, in the presence of SDS at pH 5.6, any intramolecular hydrogen bonds in bilirubin (which result in a rigid, folded configuration) will be broken and the bilirubin will exist in its linear tetraaryl structure (15, 34, 36). Under these conditions, the bilirubin ought to be oriented in the micelle such that the pyrrole nitrogen face the inner hydrocarbon core and the carboxyl groups face the solvent. Because the reactive site of the bilirubin is located in a hydrophobic region, it is very unlikely that the charged diazonium salt will be able to penetrate into the inner region of the micelle to react with the bilirubin. Consequently, we believe that the diazo coupling takes place in solution and not in the micelle. Thermodynamic considerations dictate that, as bilirubin is removed from solution by diazo coupling to form azobilirubin, more bilirubin must leave the micelle for the solvent and can then react with the diazonium salt. Because only a small amount of bilirubin will be in solution at any time, a small amount of heme can cause the observed heme interference. In addition, at the pH at which the absorbance is determined (pH 1.55), the azobilirubin can also partition into the micelles.

The small amount of precipitation that occurs upon addition of SDS to the reaction mixture in our conjugated bilirubin method is not unexpected. Putnam and Neurath (36) have shown that on the acid side of the isoelectric point, the complex between SDS and serum albumin precipitates at low concentrations of SDS. However, upon addition of more SDS, this precipitate is easily dispersed and the solution becomes clear once again.

Matrix Effects

The potential effect of the protein matrix on bilirubin studies cannot be disregarded. Although bilirubin is normally optically inactive (37, 38), circular dichroism and optical rotary dispersion measurements reveal large Cotton effects when bilirubin is bound in the presence of molar excess of human, bovine, rabbit, goat, porcine, and chicken serum albumin (37–39). Both the signs and magnitudes of these Cotton effects differ widely in these different matrices, as do the magnitudes of the apparent association constants (40). These measurements offer compelling evidence that bilirubin is bound in a coiled conformation believed to form as the molecule twists about the central methylene carbon in a relatively tight helical structure (37, 38). Because the Cotton effects change sign at about pH 5, the helical conformation is probably modified by changes in the ionization of binding groups and by pH-dependent changes in the protein conformation. The variations among the different proteins are considered to reflect subtle differences at the respective binding sites of the albumin. For the two most commonly used protein matrices in bilirubin studies, human serum albumin and bovine serum albumin, the Cotton effects in the region of 300–500 nm have opposite signs; furthermore, the binding association constant for human serum albumin is approximately five- to 10-fold that for bovine serum albumin (40, 42, 43). These conformational differences in the binding site could, together with different affinities of binding, substantially affect the ability of various accelerators to release bilirubin from serum albumin.

The circular dichroism spectra also suggest that the bilirubin-binding characteristics of serum albumin are strongly dependent upon the anion concentrations (44). Addition of anions at constant pH results in a change in the sign of the Cotton effect in the visible region, which is consistent with a change in the conformation of bound bilirubin. The anions can

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3 The critical micelle concentration is that concentration above which each additional molecule of SDS added will be incorporated into a micelle. Thus the critical micelle concentration represents the maximum monomer concentration.

4 Reynolds, J. A., personal communication.

5 Steinhardt, J., personal communication.
influence the bilirubin conformation both directly, though interaction with the charged groups that participate in the binding site, and indirectly, through interaction with distant charged groups, thereby inducing a change in the overall protein conformation.

Significant differences in the circular dichroism spectra in the visible region have been reported between charcoal-treated and untreated human serum albumin (45). Untreated human serum albumin produced an additional small band centered at 500–510 nm. These differences were attributed to possible conformational changes in the protein matrix induced by the removal of fatty acids as well as by a possible decrease in thiol content. In contrast, the absorption spectrum of human serum albumin was unaffected by treatment with charcoal. Moreover, the circular dichroism spectra of BIL-BSA showed a marked sensitivity to commercial preparations from different manufacturers, and indicated that binding characteristics are dependent on pH.

These observations have serious implications for standardization procedures and methodologic studies in bilirubin analysis and in other areas of clinical chemistry. One must rigorously specify the protein matrix composition (including the presence and concentration of ionic and fatty acids), the protein source, and the degree of pretreatment. Possible extension of these implications to other areas of clinical chemistry where proteins are present during analysis is obvious. We strongly recommend that the above-mentioned effects be carefully weighed when decisions are made in the interpretation of bilirubin-binding studies and in the preparation of protein standards for such studies.

In summary, we have developed and optimized a manual method for total bilirubin in which SDS is used as an accelerator. This method does not use caustic or viscous reagents and is free of interferences from turbidity, protein concentration, and protein matrix. HB interference is negligible at commonly encountered amounts of hemolysis (0–3.0 g/L), and is minimal up to 10 g of HB per liter. The wide range of permissible protein concentrations allows sample volumes to be chosen over a wide range under circumstances where small sample size or high sensitivity are dominant factors. The azobilirubin product has approximately a 10% higher molar absorptivity than the Jendrassik–Grob product, and the response curve for the method is linear to 250 mg of bilirubin per liter. Our method shows excellent correlation with the Jendrassik–Grob method. We have also developed a convenient method for estimation of conjugated bilirubin. The manual method should be adaptable to automated methods of analysis.

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