Biliprotein in Adult Icteric Serum—Demonstrated by Extension of the Alkaline Methanolysis Procedure

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We confirmed that the alkaline methanolysis procedure of Blanckaert ([Biochem. J. 185: 115–128, 1980]) converts the sugar conjugates of bilirubin (B<sub>S</sub>) into their corresponding methyl and dimethyl esters, which can be extracted into chloroform along with underivatized unconjugated bilirubin (B<sub>C</sub>). By this procedure, we accounted for B<sub>C</sub> nearly quantitatively, but only 76–83% of total B<sub>S</sub>. By pretreating samples containing B<sub>S</sub> and (or) B<sub>C</sub> with a caffeine/benzoate reagent, we improved the analytical recovery of B<sub>C</sub> to 85–93% without affecting the B<sub>S</sub>. When the method (+ caffeine/benzoate) was applied to adult icteric serum, a variable fraction (20–75%) of the original total bilirubin (based on diazo reactivity) remained with the protein pellet, which is routinely discarded in the original methanolysis procedure. In this pellet we demonstrated the occurrence of a strongly protein-bonded bilirubin fraction (biliprotein) similar to the recently described “delta” fraction [Clin. Chem. 28: 629–637, 1982]. The analytical and clinical implications of our findings are discussed.

Additional Keyphrases: species of bilirubin in plasma - protein binding of bilirubin - effect of caffeine/benzoate - delta bilirubin

In 1980, Blanckaert (1) described the alkaline methanolysis method for determination of bilirubins. In this method, the mono- and di-sugar conjugates of bilirubin (collectively termed “B<sub>S</sub>” here) are converted via base-catalyzed transesterification in methanol into their respective methyl and dimethyl esters. The latter are easily extracted along with underivatized unconjugated bilirubin (B<sub>C</sub>) into chloroform for subsequent quantitation. The alkaline methanolysis method appears to offer several analytical advantages over other conventional assays for bilirubin. According to Blanckaert (1), some of these advantages are: The transesterification of B<sub>S</sub> is rapid (>90% complete, with <1% saponification, within 1–2 min). The reaction products, along with underivatized B<sub>C</sub>, are extracted into chloroform with ~85% recoveries. The resulting pigments can be rapidly resolved, either by thin-layer chromatography (1) or, as later reported (2–5), by “high-performance” liquid chromatography. The bilirubin methyl esters are more readily available in pure crystalline forms than are the native sugar conjugates, so pure B<sub>C</sub> is not needed as a reference material.

In this study, our focus is on the original alkaline methanolysis method of Blanckaert (1) as applied to serum from jaundiced adults. First, we will summarize our preliminary experience in working with this method, using a serum-based matrix supplemented with B<sub>S</sub> and (or) B<sub>C</sub>. Then we will demonstrate, by extension of the original procedure, the occurrence of a firmly protein-bonded bilirubin fraction in serum from jaundiced adults.

Materials and Methods

The precautions in handling solutions containing bilirubins were as described previously (6, 7).

All chemicals used were reagent grade or better, from Kodak Laboratory Chemicals, Rochester, NY 14650, or as detailed elsewhere (6–9).

Ultraviolet and Visible Spectroscopy

This was done as reported (6) at 20–25 °C.

Serum-Based Samples and Reference Materials

Low-bilirubin (≤5 μmol/L) serum pools were supplemented with B<sub>S</sub>, dB<sub>S</sub>, or both, as described (9). Diconjugated bilirubin (dB<sub>C</sub>) was isolated from human bile and characterized as described (9). The chief detectable contaminants were water, traces of bile acids, and some (2–5%) monocojugates. Because the monoconjugates increase at the expense of dB<sub>C</sub> during storage at 0 °C or upon repeated freezing and thawing, dB<sub>C</sub> should be used only when freshly prepared. The monocojugated bilirubin (mB<sub>C</sub>) was prepared in-house from rabbit bile to >95% gravimetric purity. The strongly protein-bonded bilirubin (the “delta fraction”) was extracted from adult icteric serum as reported (7). By liquid-chromatographic analysis (7, 8), this particular preparation contained 5–8% of nondelta bilirubins. The methyl and dimethyl esters of bilirubin were obtained from Porphyrin Products, Logan, UT 84321.

Diazomethane Method

Unless otherwise stated, the Jendrassik–Gröf (J-G) assays for total bilirubin or, where indicated, for direct bilirubin were used as described (9). These were modifications according to Doumas (10).

Liquid Chromatography

Bilirubin components in serum or serum-based fluids were separated by the liquid-chromatographic method of Lauff et al. (7, 8, 11). This is not to be confused with the liquid-chromatographic system recently reported by Blanckaert et al. (2, 3, 5).

Alkaline Methanolysis Method

Unless otherwise stated, Blanckaert’s (1) method was followed closely and is referred to here as the “original procedure” or as “alkaline methanolysis.”

Modified Alkaline Methanolysis Procedure

This differs from the original procedure (1) in two main respects: (a) pretreatment of serum with caffeine/benzoate (see below) and (b) retention of the protein pellet after alkaline methanolysis. First, 0.5 mL of sample or serum was mixed with an equal volume of a caffeine/benzoate reagent (containing 50 g of caffeine, 75 g of sodium benzoate, 82 g of anhydrous sodium acetate, and 1 g of disodium EDTA per liter).
Fig. 1. Flow charts for the original and modified alkaline methanolysis procedures for serum bilirubin.

**TLC, thin-layer chromatography**

The mixture was vortex-mixed at room temperature for ~10 s, allowed to stand at room temperature in dim light (preferably under nitrogen) for 5–10 min, and vortex-mixed again for 10 s. Methanol (2 mL) was added, and the new mixture was vortex-mixed again for 10 s, then mixed with 2 mL of 20 g/L potassium hydroxide in methanol and vortex-mixed for 1 min, after which 2 mL of chloroform and then 4 mL of glycine HCl buffer, pH 2.7, were added. The mixture was gently vortex-mixed and centrifuged at 3000 rpm in a JA-20 rotor fitted in a J-21C centrifuge (Beckman Instruments, Inc., Palo Alto, CA 94304) maintained at 0–4 °C. The chloroform (lower) phase was removed and further treated as in the original procedure. The protein pellet was carefully separated from the clear aqueous medium by gentle pipetting and washed at least once with an equivalent mixture of chloroform and methanol to remove any residual B, or its methyl esters. The pellet was then dried under a stream of nitrogen and resuspended in 5 mL of a freshly prepared mixture containing, per liter, 6 mol of guanidine hydrochloride, 20 g of β-mercaptoethanol, and 0.5 mol of Tris HCl, pH 9.0. Usually, vortex-mixing of the mixture for 10 s and then sonicating it for 5 min in a Model 220 Ultrasonicator (Branson Cleaning Equipment Co., Shelton, CT 06484) at 20–25 °C was required to solubilize the pellet. With very lipemic or turbid serum, solubilization of the protein precipitate was facilitated by heating the mixture at 37 °C for 20–30 min under nitrogen.

The solution was diluted eight- to 10-fold with distilled water and ultrafiltered through a 78-mm PM-10, 10 000 dalton-cutoff membrane filter in a Model 402 stirred cell (both from Amicon Corp., Lexington, MA 02173) pressurized to 310 kPa (45 psi) with nitrogen. When the volume of the solution was reduced by fivefold, the filtrate was discarded. The fluid retained in the stirred cell was diluted again with five equal volumes of water, and the ultrafiltration was continued until the volume of the solution remaining in the cell was reduced by threefold. This procedure was repeated for three more cycles. The fluid retained in the apparatus was lyophilized, and the residue was resuspended in 0.5 mL of 50 mmol/L phosphate-buffered isotonic saline, pH 7.4, and submitted for the J-G assays of Doumas (10) or, where indicated, subjected to liquid-chromatographic analysis (11). In the latter case, the peak area was converted to micromoles of bilirubin per liter by multiplying it by the slope of a regression plot of the J-G generated total bilirubin values and the corresponding peak areas obtained with serially diluted samples of authentic protein-bonded (delta) bilirubin. The rationale for this treatment appears later in the text.

Figure 1 summarizes, in outline form, the original and our modified alkaline methanolysis procedures. Note that in later reports by Blank et al. and his co-workers (2, 3, 5), the volume of starting fluid or serum was changed to include 0.2–0.6 mL, and liquid chromatography was used in place of thin-layer chromatography.

**Direct Solution Spectrometry of Bilirubins after Methanolysis**

For samples containing only B, dBo, or mBo, the concentrations of their respective methanolysis products were estimated, either in CHCl₃ or in CHCl₃/CH₃OH (1:1 by vol), by using the molar absorptivities (ε) reported for each species in the same media (1). For mixtures of bilirubins (e.g., in sera), the total concentration of the reaction products (Bₗ) was estimated semiquantitatively by using the average of the ε values reported for B, and its methyl esters, which have closely similar absorptivities at ~445 nm in CHCl₃/CH₃OH (1:1 by vol) (1). For two reasons, we preferred this approach to that used in the original alkaline methanolysis procedure, where the reaction products were first resolved by thin-layer chromatography, then scraped off the plates and separately quantitated by direct spectrometry. First, in our hands, the pigments resolved by thin-layer chromatography (especially the methyl esters) oxidize rapidly to greenish products. Second, analytical recoveries of the pigment bands from the chromatographic plates were seldom quantitative. Therefore, we report here only the data based on the direct spectrometry of the total pigment products from sera without first separating them by thin-layer chromatography.

**Results**

**Identification of Reaction Products**

Products were identified in three ways.

First, the presumptive alkaline methanolysis products generated from authentic B, (underivatized in the process), mBo, or mBo, were carefully compared by detailed solution spectra and ε values with Bo and its corresponding authentic methyl esters from Porphyrin Products. In each case, the presumptive product was identical with the authentic reference material.

Second, we compared these same properties with those...
reported by Blanckaert (1) for the materials in either CHCl₃ alone or CHCl₃/CH₃OH (1:1), bearing in mind that the absorption maximum (λmax) and ε values of the methyl esters depend strongly on the solvent. Again, the expected products and their reference standards behaved exactly as Blanckaert had reported (1) for each solvent.

Third, the presumptive reaction products of mBₓ, dBₓ, or both in mixtures were thin-layer chromatographed as described (1) alongside methyl esters prepared independently by Porphyrin Products and by our laboratory. The dimethyl ester from the latter source was at least 95% pure according to mass spectrometry; the monomethyl ester, however, was moderately contaminated with the dimethyl ester and a trace of biliverdin, as seen on thin-layer chromatography. In all cases, the presumptive methanolysis product(s) migrated on the plate along with the predominant component in the reference standards. Again, both the putative and authentic methyl esters were unstable, often turning green when the completed plates had dried.

Estimation of Analytical Recoveries

[Bₓ]. The concentration of Bₓ recovered in the CHCl₃ phase after alkaline methanolysis of Bₓ-only samples was assessed as follows. First, the concentration of recovered Bₓ was determined by direct spectrometry, using ε = 61.5 × 10^3 L mol⁻¹ cm⁻¹ for Bₓ in CHCl₃ (1). Then the CHCl₃ was evaporated off and the concentrated pigment was redissolved in exactly the same volume of basic serum pool as before methanolysis and reanalyzed by the J-G (Doumas) method. Regression statistics for this particular plot were: slope = 0.956, intercept = −2.48, r = 0.999, averaging 77%, which contrasted with the excellent recoveries (>90%) reported by Blanckaert (1), we hypothesized, among other possibilities, that either the methyl esters were not totally extracted into CHCl₃ or the transesterification of Bₓ was incomplete. To distinguish between these possibilities, we resolubilized the protein pellets in these Bₓ fluids and reanalyzed their pigment contents by the liquid-chromatography techniques of Lauff et al. (11). We found that the pigments were largely unreacted mBₓ or dBₓ, although the absolute concentrations of these species were near the lower detection limits of the method (17–34 µmol/L). These data suggest that the transesterification of Bₓ was incomplete in the original alkaline methanolysis procedure. Because of this repeatable observation, we looked for ways to improve the recovery of Bₓ. The best method we have found is to pretreat the serum-based fluids (see the modified procedure in Figure 1) with caffeine/benzoate. This simple step improves the apparent recovery of Bₓ in different preparations from 77–80% to 85–93%.

Nature and Improved Recovery of the Firmly Bound Bₓ

Because the recovery of products with Bₓ-only samples was...
(see typical results shown in curve b, Figure 3), without affecting the corresponding recovery of B0. Using the modified procedure, we have also accounted for 90–95% of the Br (= B0 + mB + dB) from known, artificial mixtures of the three major pigments added to a serum-based matrix. Recently, using the 95% pure mB prepared in-house, we showed that the modified alkaline methanalysis procedure converted it to the monomethyl esters with ~95% recovery in the CHCl3 phase.

Application of the Original and Modified Procedures to Icteric Serum

Having ascertained the respective recoveries of B0 and Br in serum-based matrices, we decided to test both the original and modified alkaline methanalysis protocols with icteric adult sera, including samples from patients with severe hepato-biliary disorders. We soon discovered, as Blancaert et al. had reported (2, 5), that his procedure, already refined by use of liquid chromatography (2, 5), gave substantially lower estimates of total bilirubin in these sera than did different diazo tests. This underestimate by the alkaline methanalysis procedure relative to the J-G method, which varied between 28% and 80% for various sera, was only moderately decreased (to 20–75%) when we pretreated the same samples with caffeine/benzoate according to our modified procedure. This observation led us to conjecture that either the diazo methods overestimate the true serum bilirubin—as suggested by Blancaert et al. (2)—or the procedure of alkaline methanalysis (+ caffeine/benzoate) was not detecting a sizable fraction of the total bilirubin in many icteric sera. Around this time, Lauff et al. (7, 8, 11) devised a liquid-chromatography protocol that clearly separates bilirubins in adult serum into four chief fractions. These are, in order of increasing polarity: B0 (α fraction), mB (β fraction), dB (γ fraction), and a firmly protein-bonded fraction, first called “δ bilirubin” by Kuenzle et al. (12) and recently verified by us to be a biliprotein2 (7, 8). This background information, and our observation that the protein pellets from many adult sera are yellow even after five washings with CHCl3 after methanalysis, led us to focus attention on the neglected protein-pellet phase. Our experiments in this regard were in three stages.

Stage one. At this stage, we explored the behavior of authentic biliprotein in the alkaline methanalysis protocol. A concentration series of the biliprotein isolated as described (7) was subjected to the alkaline methanalysis method modified as shown in Figure 1. In each case, 80–90% of the original bilirubin, based either on the diazo reactivity (7) or on liquid-chromatographic peak area (10), was recoverable in the resolubilized protein pellet. The recovered material behaved like the original biliprotein in its major physicochemical properties (7, 8). About 5 to 10% of the original pigments appeared in the CHCl3 phase, and these were identified on thin-layer chromatography as being principally the methyl and dimethyl esters; a smaller fraction of the original biliprotein (<5%) was detectable in the aqueous phase after the protein pellet was removed. These data showed that the major bile pigment in authentic biliprotein behaves distinctly differently from either B0 or Br in the alkaline methanalysis procedure.

Stage two. In the second stage, we investigated any possible interactions among B0, Br, and the biliprotein during methanalysis. To do this, we dissolved freshly prepared biliprotein in a serum-based matrix containing gravimetrically known and diazo-confirmed (9) concentrations of B0 and (or) dB. In this test, the highest concentration of the protein-linked bilirubin was 94.2 µmol/L.3 In this experiment, we observed that B0 and its conjugates were 91–94% accounted for analytically in the CHCl3 phase, whereas ~98% of the biliprotein was found in the pellet phase. Thus, the presence of various proportions of the different bilirubin fractions in known mixtures does not appear to affect their respective recoveries as a result of alkaline methanalysis.

Stage three. To illustrate this stage of investigation, we sampled 11 jaundiced sera from nine adults. As shown in Table 1, these patients differed in their primary diagnoses, total bilirubin values (as measured by the J-G method), and in the nominal compositions of the four bilirubin subfractions (based on liquid-chromatographic peak areas). Each sample was divided into two or more identical volume aliquots and subjected to our modified alkaline methanalysis procedure. The resulting resolubilized and ultrafiltered protein pellets were analyzed, first qualitatively, then semiquantitatively, as follows.

(a) Qualitative analysis of protein fractions isolated after alkaline methanalysis: The protein fractions isolated from patients’ sera after the modified alkaline methanalysis procedure were compared directly with the authentic biliprotein for their liquid-chromatographic behavior; diazo-test reactivities (total vs direct); solution spectra; electrophoretic mobilities; isoelectrofocusing patterns; and their relative resistances to acid, base, detergents, and different digestive enzymes. The detailed conditions for these analyses are described elsewhere (7). In all instances, the two types of protein isolated behaved similarly. In two sera with nominal concentrations of the protein-bonded bilirubin fraction ≥50% of the total bilirubin (see Table 1), the resolubilized and ultrafiltered protein fractions were further purified as described (7) to remove most of the globulins. Again, the resulting albumin-rich fractions were largely indistinguishable from authentic biliprotein, based on the criteria above. These observations collectively showed that the protein isolated by the modified alkaline methanalysis procedure contains a similar biliprotein component as realized by the procedure of Lauff et al. (7). Consistent with all our findings to date, this biliprotein is most probably a strong covalent complex between bilirubin and albumin or an albuminlike protein. Accordingly, we decided to use the same procedures (namely, the J-G and liquid chromatography as defined in Materials and Methods) to estimate the bilirubin content in the biliprotein fractions prepared by both routes (modified alkaline methanalysis and method of Lauff et al.).

(b) Semiquantitative analysis of the isolated biliprotein fractions: Figure 4 shows that when the protein pellet fraction was deliberately omitted from the alkaline methanalysis protocol (+ caffeine/benzoate), the latter was severely negatively biased relative to the J-G diazo method. By contrast, when the protein-bonded fraction was included in the alkaline methanalysis protocol (Figure 5), the latter became much more highly correlated with its diazo counterpart. Figure 6 further illustrates that the percent bias between the J-G assay and the alkaline methanalysis method (minus the protein fraction) correlated with the biliprotein content in all 11 patients’ sera tested. These data, considered together, showed

2 We prefer “biliprotein” or “protein-bonded bilirubin” to the term “delta bilirubin” or “δ fraction” because the former names imply a strong, possibly covalent complex between bilirubin and protein whereas the latter may be confused with the δ ring of the porphyrins or the δ-diazo fragment of bilirubins.

3 We refrained from increasing the protein-linked bilirubin concentration in the test fluids because this would mean dissolving more of the biliprotein, which, as isolated (7), contains most of the original serum albumin. This would in turn artificially increase the final protein concentration (to ≥120 g/L) at the expense of recovery in either the CHCl3 or the pellet phase (see stage one).
that the diazo-positive, strongly protein-bonded bilirubin fraction is the major cause of the severe negative bias observed between the two methodologies. Finally, Figure 7 shows that the protein-bonded bilirubin fraction as estimated by our modified alkaline methanolysis procedure correlates with that given by the liquid-chromatographic technique of Lauff et al. (7). Note that the protein-bonded bilirubin can vary from \( \sim 20 \) to 75% of the total bilirubin values, based on our modified procedure; this is also the range observed from a wider sampling of jaundiced adult sera (not shown).

Discussion

Our purpose here was to gauge the performance of the alkaline methanolysis procedure first described by Blanckaert (1), especially as it related to icteric serum from adults. Our findings may be summarized as follows:

(a) Using authentic samples of \( \text{B}_0 \), \( \text{B}_s \) and (or) the recently characterized protein-bonded bilirubin (7), we observed that, after methanolysis, underivatized \( \text{B}_0 \) is recovered nearly quantitatively, 76–83% of \( \text{B}_s \) is accounted for, and the protein-bonded fraction at less than \( \sim 10\% \), in the \( \text{CHCl}_3 \) phase.

(b) By pretreating the serum-based solution with a caffeine/benzoate reagent we improve the apparent recovery of \( \text{B}_s \) (where \( \text{B}_s = \text{mB}_s + \text{dB}_s \)) to 85–93% without materially affecting the recoveries of the other pigment fractions. The mechanism of this improvement is unknown and warrants further investigation. Although there may be other reasons for the different recoveries of \( \text{B}_s \) reported by us and by Blanckaert (1), we have presented direct evidence for incomplete transesterification of \( \text{B}_s \) under conditions of the original alkaline methanolysis protocol.

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**Table 1. Bilirubin Profiles in Randomly Selected Sera from Adult Patients**

<table>
<thead>
<tr>
<th>Disease category</th>
<th>Total bilirubin (( \mu \text{mol/L} ), based on J-G [Doumas] (9, 10))</th>
<th>( \alpha ) Unconjugated bilirubin</th>
<th>( \beta ) Monoconjugated bilirubin</th>
<th>( \gamma ) Diconjugated bilirubin</th>
<th>( \delta ) Protein-bonded bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive heart failure (sequential draws)</td>
<td>202.2</td>
<td>21</td>
<td>37</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Adenocarcinoma, metastasis</td>
<td>124.9</td>
<td>19</td>
<td>19</td>
<td>14</td>
<td>48</td>
</tr>
<tr>
<td>Dubin–Johnson syndrome</td>
<td>80.9</td>
<td>20</td>
<td>16</td>
<td>21</td>
<td>49</td>
</tr>
<tr>
<td>Sepsis, pancreatitis</td>
<td>35.7</td>
<td>29</td>
<td>30</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Metastatic prostatic cancer</td>
<td>108.9</td>
<td>42</td>
<td>9</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>31.0</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>77</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>292.8</td>
<td>55</td>
<td>28</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Resolving hematomata</td>
<td>142.1</td>
<td>10</td>
<td>25</td>
<td>13</td>
<td>53</td>
</tr>
<tr>
<td>After cardiac surgery</td>
<td>96.5</td>
<td>31</td>
<td>32</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>56.6</td>
<td>35</td>
<td>27</td>
<td>14</td>
<td>23</td>
</tr>
</tbody>
</table>

* These sera, selected without conscious bias, illustrate the highly variable bilirubin concentrations and compositions encountered in many jaundiced adults. Because there could be undiagnosed secondary disorders and other unidentified factors involved, we caution against any attempt to correlate a particular disease category with any bilirubin profile based on what is tabulated here. Such clinical correlations should await much wider sampling of patients, not only of clearly diagnosed pathologies but also at different stages of the diseases.
(c) Most important, we found that, by resolubilizing the protein pellet that is routinely discarded in the original method (1), we could demonstrate the occurrence therein of a strong, possibly covalent bilirubin–protein complex that behaves like the authentic biliprotein fraction isolated (7) by a different method. We hope to confirm this by comparing the amino acid sequence of the pigment-binding region in the biliproteins obtained by both routes. Analytically, the bilirubin content in this fraction appears to account largely for the negative bias elicited when the original alkaline methanolation (± caffeine/benzoate) was compared with, e.g., the J-G diazo method (Doumas) (10) for many icteric sera from adults. Because 5 to 10% (highest percentage seen, ~12%) of the authentic protein-bonded pigment appears in the CHCl₃ phase as methyl esters, we cannot immediately exclude the possibility that a relatively small fraction of the original protein-linked pigment is available for alkaline methanolation. This issue might have to be settled by using totally purified protein-bonded fraction free of any extraneous proteins (i.e., those unrelated to the biliprotein) or tightly adsorbed bilirubins.

The fact that the protein-bonded bilirubin can be demonstrated independently by the method of Lauff et al. (7) and by our modified alkaline methanolation procedure in the same adult sera lends further credence to the reality of this entity in serum. Because that fraction in serum can be 20 to 75% of the total bilirubin (present study) in different jaundice diseases, this entity might have hitherto-overlooked clinical significance. Parallel studies will explore this theme in greater depth. To date, the only icteric sera in which the biliprotein appeared to occur at <5–8% of the total bilirubin were those from newborns (<14 days old) (13), adults with hemolytic jaundice, and certain genetic disorders (8).

Although our modified alkaline methanolation protocol represents an important extension of the original method, it is at this stage semiquantitative and not readily adaptable to routine clinical use because physical recovery of the denatured protein and its preparation for subsequent analysis are neither simple nor rapid. Also, there is no simple or definitive assay for the different bilirubin subfractions in serum. Therefore, some of the correlations presented (e.g., Figures 6 and 7) should be used only to show overall trends, not precise quantitative relationships between different methods. Future work will be aimed at refining different approaches towards quantitating the protein-bonded bilirubin fraction from serum.

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10. Doumas, B. T., personal communication to W. D. Fellows (1978); see also ref. 9.