Isolation and Preliminary Characterization of a Fraction of Bilirubin in Serum That Is Firmly Bound to Protein

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We have isolated from pathological sera a bilirubin fraction (δ) that is very tightly, if not covalently, bound to protein, most likely albumin. This δ fraction absorbed at a λmax of 433 nm in the visible spectrum, between the λmax of unconjugated (α) and that of conjugated (B) bilirubin when measured in solutions containing albumin. However, unlike the other bilirubin species, this fraction could not be separated from the proteins in serum by exhaustive ultrafiltration in the presence of caffeine/benzoate solution. In the Jendrassik–Grop diazo procedure for bilirubin analysis, the δ fraction gave a large direct reaction (78–89% of the total reaction). Yet, when relatively hydrophobic azo dyes were formed by reaction of the δ fraction with the diazoammonium salt of dichloroaniline, only 50% of the dyes were extractable from aqueous solution. On chromatography, the rest remained associated with protein. Of the extractable dye, more than 70% was accounted for by two liquid-chromatographic peaks with retentions identical with those of azo dyes formed from unconjugated bilirubin. This δ fraction was not appreciably separated from protein by treatment with strong acid or base, or by prolonged digestion with various enzymes. Finally, in a highly denaturing solvent (urea/mercaptoethanol), this fraction was not dialyzable through a membrane with a 12 000-dalton cutoff.

Additional Keyphrases: evidence for covalent binding • species of bilirubin in plasma • evidence that the bound fraction is heterogeneous • isolation procedures • binding by albumin

Recently a “high-performance” liquid-chromatographic (HPLC) procedure (1) was developed for separating bilirubin species in serum. This technique is similar to that of Kuenzel and co-workers (2–7) in that serum is not totally deproteinized before chromatography. Four distinct bilirubin fractions in serum were reproducibly separated. The retentions of three of these fractions corresponded to those of unconjugated (α), monoconjugated (β), and dicongjugated (γ) bilirubin, and the fourth (δ) eluted always coincidentally with albumin. Because the other bilirubin species are separated from their complexes with protein by interaction with the HPLC packing material, it was evident that the association of this fourth fraction (δ) with protein was fundamentally different.

Earlier, Kuenzel and co-workers (2–7), using an open-column chromatographic procedure, also observed in most pathological sera a fraction of bilirubin tightly bound to protein. Since then, little work has been published on the nature of this fraction (δ). Yet, it appeared likely that the existence of a bilirubin fraction very firmly bound to protein in serum might explain the numerous observations that some bilirubin is usually irretrievably lost whenever proteins are precipitated from serum (9–13), particularly if the serum contains a high proportion of direct-reacting bilirubin species as determined by diazo tests (9, 13).

We now present evidence based upon HPLC, ultrafiltration, dialysis, electrofocusing, gel electrophoresis, and chemical tests, which is consistent with covalent bonding of one or more bilirubin species to albumin.

Materials and Methods

All of the operations on solutions containing bilirubin species were carried out under subdued light, or preferably under yellow-filtered light. Whenever possible, solutions were degassed under a stream of helium before contact with solutions containing bilirubin species. Alternatively, a nitrogen-rich atmosphere was used, e.g., for enzymic digestions. In addition, whenever practicable, long (overnight) operations such as ultrafiltration or dialysis were carried out in darkness and at low temperature (4 °C). Unless otherwise stated, all chemicals were reagent grade or better, from Kodak Laboratory Chemicals, Rochester, NY 14650. Ammonyx-LO (dodecyldimethylamine oxide) was obtained from Onyx Chemical Co., Jersey City, NJ 07302. All nonproteolytic, degradative enzymes were supplied by Sigma Chemical Co., St. Louis, MO 63178, or Calbiochem, La Jolla, CA 92037.

Isolation of δ fraction of bilirubin: For the isolation of the δ fraction of bilirubin, caffeine/benzoate solution was prepared at the concentration used as an accelerator in the Jendrassik–Grop diazo procedure for bilirubin analysis (14). Pathological sera, each with a total bilirubin value of 80–300 mg/L by a modified Jendrassik–Grop analysis similar to that described by Doumas (15) and each with a direct value at least 50% of the total value, were pooled to give 5–10 mL of sera. The pool was diluted with an equal volume of distilled water and then with a volume of the caffeine/benzoate reagent equal to 12-fold that of the initial volume of the pool. The sample was then ultrafiltered through a 76-mm PM10, 10 000-dalton cutoff membrane filter in a Model 402 stirred cell (both from Amicon Corp., Lexington, MA 02173) pressurized to 311 kPa (45 psi) with helium gas. When the volume of the solution had been reduced by a factor of seven, the filtrate was discarded, another aliquot of the caffeine/benzoate reagent equal to six times the volume retained behind the membrane was added to that in the apparatus, and ultrafiltration was continued. This procedure was repeated for four or five cycles. Residual caffeine/benzoate solution was washed from the sample by diluting the retained fraction to its initial volume with distilled water and continuing ultrafiltration. This was repeated four or five times to free the retained fraction from most of the excess caffeine/benzoate. The fraction was then transferred by pipet to a beaker for precipitation of the globulins.

Anhydrous sodium sulfate (138.5 g; MCB Reagents, Norwood, OH 45212) was dissolved in ~400 mL of hot water and adjusted to pH 7.0 ± 0.2 with dilute sodium hydroxide and dilute hydrochloric acid. At 37 °C the solution was diluted to volume in a 500-mL volumetric flask and kept at 37 °C in a water bath. A 14-fold volume excess of this solution was added slowly (2–3 min), with gentle stirring, to the retained fraction of the caffeine/benzoate-treated pool of sera. The precipitated globulins were removed from this solution by filtration.


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through a 0.45-μm microporous filter (Amicon Corp.). The precipitate was discarded, and the filtrate was diluted with two volumes of distilled water and then passed through a fresh Amicon PM10 membrane filter. When the volume of the solution was reduced to <50 mL, 200–300 mL of distilled water was added to the retained material, and ultrafiltration was continued. This procedure was repeated four or five times to remove most of the sodium sulfate from the isolated δ fraction.

The final solution, concentrated usually only to twice the initial pool volume, was filtered through another 0.45-μm filter if it was cloudy, and then either stored frozen at −4 °C or lyophilized first and then stored in a freezer.

**Unconjugated bilirubin (α)–human serum albumin (HSA) for ultrafiltration:** A sample of unconjugated bilirubin plus HSA (Cohn Fraction V), both from Sigma Chemical Co., was prepared by dissolving 1.65 mg of unconjugated bilirubin in 0.5 mL of 0.1 mol/L sodium carbonate with ultrasonic mixing. When solution appeared to be complete, 20 mL of an aqueous solution of HSA (30 g/L) was added. Dilute HCl was added to adjust the pH to 7.0, and the solution was diluted to volume with distilled water in a 25-mL volumetric flask.

**HPLC separation of bilirubin components in serum:** HSA was dissolved to give 100 g/L in a solution of ascorbic acid (20 g/L) and sodium phosphate (0.1 mol/L, pH 6.7), which was then adjusted to pH 7.7 ± 0.2 with dilute NaOH. Serum (0.25 mL) was diluted with an equal volume of the HSA/ascorbic acid solution and allowed to set for 20 min in a sealed vial at 37 °C in a water bath. Sodium sulfate solution (7 mL; prepared as described above) was added to the vial with gentle shaking. The vial was resealed and heated for ~2 min at 37 °C. The precipitated globulins were removed from the treated sample by filtration through a 0.45-μm filter (Millipore Corp., Bedford, MA 01730) overlaid by a fiber prefilter (Type A-E, 25 mm; Gelman Instrument Co., Ann Arbor, MI 48106) in an Amicon MMC ultrafiltration unit pressurized by helium. The filtrate was collected in a 25-mL volumetric flask containing 1.5 mL of 0.5 mol/L sodium phosphate solution and 100 g of ascorbic acid per liter (pH 5.5). To the precipitate in the filtration unit, 0.25 mL of the HSA/ascorbic acid solution and 0.25 mL of distilled water were added, followed by 7 mL of sodium sulfate solution. The solution was stirred rapidly for 1 min, filtered through the precipitate, and combined with the first aliquot in the flask. The sample was diluted to volume with distilled water.

For samples that did not require protein separations, such as bilirubin/albumin standard solutions or isolated fractions of the bilirubin conjugates (Bδ) or of the δ component, 0.25 mL of sample was added to a 25-mL volumetric flask containing 0.5 mL of the HSA/ascorbic acid solution. Then 1.5 mL of the buffered 100 g/L ascorbic acid solution was added, followed by 14 mL of the sodium sulfate solution, and the sample was diluted to volume with distilled water.

For HPLC separation, aliquots of diluted and sulfate-treated samples (0.5 or 0.8 mL) were injected onto a reversed-phase column (RP-10A; Brownlee Labs, Santa Clara, CA 95050) maintained at 41 °C. The bilirubin fractions were separated by gradient-elution chromatography. The chromatographic system and separation have been described in detail elsewhere (1).

**Dialysis as evidence of covalent bonding:** Unconjugated bilirubin (α) was dissolved in a 50 g/L HSA solution in the same manner as that given for the ultrafiltration experiment, except that, after neutralization, the sample was diluted to volume with the HSA solution. Aliquots of this sample (bilirubin concn, 28.4 mg/L) and of the isolated δ fraction were lyophilized for 48 h, in the dark. Samples of 50 mg each of the lyophilized unconjugated-bilirubin/albumin preparation or of the lyophilized δ fraction were then dissolved in 3 mL of 50 mmol/L Tris HCl buffer at pH 9.1 containing, per liter, 0.2 mol of mercaptoethanol and 8 mol of urea. In dialyzer tubing with a molecular-mass cutoff of 12 000 daltons (type 8667-A; Fisher Scientific Co., Pittsburgh, PA 15219), each of the dissolved samples and a blank consisting of 50 mg of albumin in the same solvent were dialyzed without agitation for 43 h in the dark at 4 °C against 10 mL of the same buffer. The absorbance of the filtrates and retentates at 440 nm for unconjugated bilirubin and that at 424 nm for the δ fraction and the blank were measured in 1-cm semimicro ultraviolet cells (Fisher Scientific Co.) in an ultraviolet/visible spectrophotometer (Model 552; Perkin-Elmer Corp., Norwalk, CT 06856).

**Purification of bilirubin diglucuronide by HPLC:** Bilirubin conjugate, Bα, was isolated from human bile by the modified Lucassen procedure of Wu et al. (16). The material was stored in a freezer at −4 °C until immediately before HPLC purification, when a 1 g/L solution was made in sodium acetate buffer (2 mol/L, pH 4.75). By means of a loop injection valve (Model 7120; Rheodyne Inc., Berkeley, CA 94710), 200 μL of sample was injected into an HPLC system consisting of two reciprocating pumps (Model 6000A; Waters Associates, Milford, MA 01757), a gradient programmer (Model 660, Waters Associates), a high-performance reversed-phase column (RP-10A; Brownlee Labs, Santa Clara, CA 95050), and a variable-wavelength spectrophotometer (Model LC-55, Perkin-Elmer Corp.). Components were separated by elution for 12 min at 2.0 mL/min with a mobile phase consisting of absolute ethanol–methanol–0.02 mol/L sodium acetate buffer (pH 4.75), 5/43/52 by vol. Bilirubin species eluting from the column were detected by their absorbance at 455 nm in the spectrophotometer. The bilirubin diglucuronide eluted in 7.5 min; after passage through the detector it was collected in a brown vial under a stream of nitrogen, diluted with an equal volume of distilled water, and immediately frozen in a solid CO2/acetone bath, still under nitrogen. The impurities present in the original sample were retained by the column under these conditions but were washed from the column by elution for 3 min with a final mobile phase consisting of the above three components in the volume ratio of 5/86/9. Both mobile phases and all solvents or reagents that came into contact with the diglucuronide were degassed with helium just before use.

After the HPLC column was regenerated for 5 min with the initial mobile phase, succeeding Bα aliquots were similarly separated and collected. All fractions were pooled before further workup.

Bilirubin diglucuronide was separated from the pooled collections by extraction with a Waters C18 Sep-Pak cartridge (Model 51910, Waters Associates). Before use, the Sep-Pak was rinsed with successive 10-mL portions of distilled water, a 10 g/L solution of ascorbic acid adjusted to pH 4.75 with dilute NaOH, methanol, and distilled water, all of which had been helium purged and kept near 0 °C. The collected sample was allowed to thaw and, while still cold, was passed through the Sep-Pak, followed by 10 mL of ice water. The retained bilirubin diglucuronide was eluted from the Sep-Pak by passing 3 mL of ice-cold methanol through it with a syringe. The solution was collected in a vial and methanol was evaporated from it by use of a vacuum pump (Model 150; Precision Scientific Co., Chicago, IL 60647). Until needed, the solid diglucuronide was stored at liquid-nitrogen temperature.

**Formation of azo dyes with 2,4-dichloroaniline:** The preparation of azo dyes from bilirubin species and dichloroaniline was adapted from the method of Rand and diPasqua (17). The dichloroaniline reagent was used at only 1 g/L, and the diazotized reagent was diluted with five volumes of distilled water rather than methanol. Unconjugated bilirubin was dissolved in HSA solution (50 g/L) in a manner similar to that of Routh (18). Bilirubin diglucuronide, purified as described above, was dissolved directly in HSA solution (50 g/L), and
the δ fraction was used directly as isolated from pathological sera.

The reaction was carried out by successively adding to 0.25 mL of sample: 1.0 mL of water, 2.0 mL of dimethyl sulfoxide, and 1.0 mL of the diazotized and diluted dichloroaniline reagent. After 10 min, the solution was passed by means of a syringe through a Waters C18 Sep-Pak, which had been prepared by successive rinses with 10 mL each of distilled water, methanol, and distilled water. Residual reagent was washed from the Sep-Pak by 10 mL of water. The retained dyes were eluted by passing 3 mL of methanol through the Sep-Pak. The methanolic solution was taken to dryness in a vial under reduced pressure. The solid dyes were stored at −4 °C.

**HPLC separation of azo dyes formed from diazotized 2,4-dichloroaniline:** The chromatography apparatus used to separate the azo dyes was identical with that described above for the purification of bilirubin diglucuronide except that a C18 reversed-phase column (Partisil C18 Type 2 with 15% loading, 4.6 mm i.d. × 25 cm long; Whatman Inc., Clifton, NJ 07014) was used for the separation, and the spectrophotometer was set at 546 nm to detect the azo dyes. After dissolution in an equivolume mixture of methanol and sulfuric acid (0.2 mol/L, pH 4.75), 200-μL aliquots of the azo dyes were injected into the column by means of the loop injector. The dyes were separated by elution at 2.0 mL/min with a 10-min linear gradient from an initial mobile phase consisting of acetonitrile/ethyl acetate/methyl acetate/distilled water (10/11/15/42 by vol) with 1.4 mL of Waters Pic A reagent per 100 mL to a final mobile phase consisting of the initial mobile-phase constituents in the volume ratio of 10/11/15/35, again with 1.4 mL of Waters Pic A reagent added per 100 mL of mobile phase.

**Electrofocusing and gel electrophoresis:** Electrofocusing and gel electrophoresis were performed in an LKB 2117 Multiphor unit (LKB Instruments Inc., Rockville, MD 20852) according to the manufacturer's instructions. The HSA marker was a fatty-acid-free preparation of Cohn Fraction V (Miles Labs., Inc., Elkhart, IN 46515). The human hemoglobin was an in-house preparation, checked for purity by spectrophotometry and by SDS-gel electrophoresis. The PI markers were supplied by U.S. Biochemical Corp., Cleveland, OH 44122.

For diazo staining of the electrofocused gel, the section of gel reserved for this purpose was sprayed with a diazo reagent prepared by mixing reagents A and B exactly as described by Routh (14). Within 1–2 min of the spraying, the azo spots appear. In all instances, the diazo-positive spots or bands correspond identically to those appearing as yellow spots after the electrofocusing runs. The electrophoresed gels could be similarly stained.

**Thin-layer chromatography:** Thin-layer chromatography of the δ fraction after different types of treatment (e.g., acid, base, methanol) was done on silica gel G-60 plates with chloroform/methanol/water (24/14/3 by vol) as described by Blumenthal et al. (19). In some runs the solvent system also contained, per liter, 10 g of one of the denaturants listed below in Table 3.

**Enzymic digests:** Portions of the lyophilized δ fraction were dissolved in Tris HCl buffer (50 mmol/L, pH 8.0) to give a final concentration of 10–50 mg of total bilirubin per liter, as determined by Jendrassik–Grob analysis (15). These were incubated with 0.5–2.5 g of Streptomyces griseus protease (Pronase CB; Calbiochem-Behring Corp., San Diego, CA 92122) per liter or with other proteases for 2–4 h at 37–40 °C, under nitrogen and subdued light. After incubation, the enzymic digest was immediately cooled to 0–4 °C. Any insoluble material was removed by centrifugation at 5000 X g for 15 min at 0–4 °C. In general, any precipitated material (usually a trace amount) did not contain any diazo-positive or yellow components. The yellow supernate or the clear incubated mixture was concentrated by lyophilizing or immediately dialyzed as indicated in the text. For the other enzymes referred to in the Results section, the incubations were conducted under conditions similar to those described for proteases, except that the exact pH used in each case was at or near that recommended by its supplier as optimal for the enzyme. The temperature was 37 °C. Unless otherwise noted, all nonproteolytic degradative enzymes used were tested at two separate final concentrations, 1 and 5 g/L.

**Dialysis in the presence of other denaturants:** The δ fraction in the presence of a series of protein denaturants (see Table 3) was dialyzed in Spectrapor membrane tubing (3500-dalton cutoff) from Spectrum Medical Industries, Inc., Los Angeles, CA 90064. The tubing was boiled in sodium bicarbonate solution (10 g/L) for about 15 min, then in distilled water for another 15 min, and finally rinsed thoroughly with de-ionized distilled water. The incubation mixtures were dialyzed against water at 0–4 °C for at least 12 h. Absorbances of the dialysates (the fraction retained in the dialysis bag) and of the dialysate at 280 nm (for protein) and 450 nm (for bilirubin) were monitored against appropriate blanks in a Perkin-Elmer 576 ultraviolet–visible spectrophotometer at room temperature.

**Gel filtration:** For gel filtration experiments with enzymically treated aliquots of the isolated δ fraction, we used small columns (0.5 cm i.d. × 5–7 cm long) of Sephadex G-75 gels (Pharmacia, Uppsala, Sweden) in 50 mmol/L Tris HCl buffer at pH 7.6 and were monitored at both 280 nm for proteins and peptides and 450 nm for bilirubin species against blank buffer in a Perkin-Elmer 576 ultraviolet–visible spectrophotometer.

**Results**

**HPLC separation of bilirubin species in serum:** In most pathological sera, four distinct bilirubin fractions were separated by the HPLC procedure given above. Figure 1 shows a typical separation obtained for a pooled sample of sera with above-normal concentrations of both direct and total bilirubin. The two traces were obtained concurrently for the same sample with two spectrophotometers connected in series at the outlet of the HPLC column. The first, monitoring absorbance at 450 nm, responded primarily to bilirubin species, and the second, set at 280 nm, responded primarily to proteins. The notation in the figure is taken from the work of Kuenzle and co-workers (2–7), who earlier had used an open-column chromatographic procedure to separate bilirubin in serum into four fractions: α—unconjugated bilirubin, β—monocojugated, γ—diconjugated, and δ, a fraction more strongly attached to protein than the others, yet yielding predominantly a "direct" response in the standard diazo procedures for bilirubin analysis.

The identification of the α and γ fractions in the chromatogram was confirmed by the separation of commercially available unconjugated bilirubin in albumin solution as well as samples of bilirubin diglucuronide in albumin solution, purified and prepared as described in the Materials and Methods section. Both of these preparations had retention times consistent with the labeling of the fractions in Figure 1. The β fraction, which eluted after the diglucuronide but well before the unconjugated fraction, showed the intermediate polarity in the chromatograms expected for a monoconjugate. This is also the predominant fraction in rabbit bile and in bile obtained from pediatric patients but is subordinate to the diconjugate in adult human bile that we have separated by the HPLC procedure (1). In addition, when serum samples were allowed to stand at room temperature for 14–48 h, the di-conjugate (γ) (as observed by HPLC) decreased in concentration, while the monoconjugate (β) at first increased and
then decreased as the hydrolysis proceeded and the unconjugated (α) material became predominant.

Isolation of δ fraction of bilirubin: The δ bilirubin fraction from the sera used for the chromatogram in Figure 1 yielded only one major peak by HPLC, which eluted with the same retention time as that for albumin (Figure 2). In the isolation procedure, caffeine/benzoate reagent was used in excess and at a concentration that disrupted the complex between albumin and unconjugated bilirubin as well as the complexes between the conjugates and albumin. The association between protein and bilirubin in the δ material was not similarly affected. The isolation procedure took advantage of this difference in protein-binding strength so that only the δ fraction was retained with the proteins (M_r > 10,000).

A 9-mL sample of unconjugated bilirubin at 66 mg/L in HSA solution (30 g/L) was ultrafiltered under conditions identical with those used for the isolation of the δ fraction from pathological sera. Figure 3 shows the HPLC separations (monitored at 450 nm) of the starting material and the final retained material after ultrafiltration. No unconjugated bilirubin remained with the retained HSA, and there was no evidence for formation of the δ fraction. The small peak at the retention time for HSA was attributable to the albumin itself, which was slightly yellow. Background absorbances of this magnitude are observed for all samples containing HSA.

Visible spectroscopy and reference azo analysis: That the δ fraction is a bilirubin derivative can be inferred from both its visible spectrum and its response in the Doumas et al. (15) version of the Jendrassik–Grof diazo procedure for bilirubin analysis. The visible spectrum was similar to those spectra obtained for the conjugated bilirubin (B_c) and unconjugated bilirubin (α) in buffered albumin solution (Figure 4). Unconjugated bilirubin absorbed maximally at 459 nm, and the B_c material, which contained both mono- and diconjugates, absorbed maximally at 422 nm; the δ fraction absorbed maximally between the two, at 433 nm. With several different isolations of the δ component from different pools of pathological sera, values for total and direct bilirubin were obtained (Table 1) by a modification of the Jendrassik–Grof method for bilirubin analysis given by Doumas et al. (15). The frac-
Table 1. Bilirubin Analysis of Isolated δ Fraction from Various Pools of Pathological Sera

<table>
<thead>
<tr>
<th>Isolation</th>
<th>Total mg/L</th>
<th>Direct mg/L</th>
<th>% Direct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (frozen)</td>
<td>8.9</td>
<td>7.5</td>
<td>84</td>
</tr>
<tr>
<td>2 (frozen)</td>
<td>9.9</td>
<td>8.7</td>
<td>88</td>
</tr>
<tr>
<td>3 (frozen)</td>
<td>24.2</td>
<td>20.2</td>
<td>83</td>
</tr>
<tr>
<td>4 (lyophilized)</td>
<td>20.3</td>
<td>17.8</td>
<td>88</td>
</tr>
<tr>
<td>5 (frozen)</td>
<td>8.9</td>
<td>7.4</td>
<td>83</td>
</tr>
<tr>
<td>6 (frozen)</td>
<td>45.5</td>
<td>34.5</td>
<td>76</td>
</tr>
<tr>
<td>7 (lyophilized)</td>
<td>16.2</td>
<td>14.4</td>
<td>89</td>
</tr>
</tbody>
</table>

This was not the case. The absorbance of the reaction mixture, measured at 530 nm before and after passage through the Sep-Pak, was only halved (±10%) by passage through the Sep-Pak. The fraction of dye that passed through the Sep-Pak was diluted with water and ultrafiltered through a membrane that has a 10,000-dalton cutoff. The dye remained with the retained protein. It was chromatographed in the HPLC system we used to separate bilirubin fractions in serum, and it eluted at the same retention time as albumin and the native δ fraction (Figure 5). Thus, even after formation of azo dyes that cause the bilirubin itself to split into two dipyrrolic fragments, some bilirubin residue(s) and protein remained tightly associated.

Figure 6 shows typical chromatograms obtained with the second HPLC system for the separation of the azo dyes generated from unconjugated bilirubin and bilirubin diglucuronide after reaction with diazotized 2,4-dichloroaniline, as well as the Sep-Pak extractable dyes after reaction with the δ fraction. For unconjugated bilirubin, two dyes (presumably isomers) were observed, and for bilirubin diglucuronide there

![Figure 4: Visible spectroscopy of bilirubin species](image)

![Figure 5: HPLC separation of water-soluble azo dyes formed from the reaction of diazotized 2,4-dichloroaniline with the δ fraction](image)
were two different dyes, which eluted earlier. In this reversed-phase HPLC system, earlier elution is generally associated with increased water solubility, as should be the case for azo dyes containing a glucuronide conjugating group. Most of the dyes from the δ fraction that were retained on the Sep-Pak, however, had retention times identical with those for unconjugated bilirubin. More than 70% of the separated dyes eluted at the retention time of these two dyes; the remainder eluted as a multiplicity of smaller peaks with retention times between those for the dyes containing glucuronide-conjugating groups and the dyes from unconjugated bilirubin.

Dialysis in urea/mercaptoethanol: Table 2 lists the absorbances monitored for the dialysis in urea/mercaptoethanol of samples containing unconjugated bilirubin and albumin, albumin alone, and the isolated δ fraction. After 43-h dialysis, the final values inside and outside the dialysis tubing for the unconjugated-bilirubin/albumin sample were not yet equal, presumably because, without stirring, the solutions had not reached equilibrium. However, the relatively large value observed for the solution outside the tubing showed that in this system, even with excess albumin present, unconjugated bilirubin was readily dialyzable through the 12,000-dalton-cutoff membrane. The blank values for the albumin solution inside the tubing were consistent with its not being dialyzable. The small increase in absorbance noted for the solution outside the tubing may indicate some small proportion of dialyzable impurities in the albumin itself or in the dialysis tubing. Likewise, the minor absorbance observed for the solution outside the tubing for the δ fraction did not indicate that the isolated material was readily dialyzable, but that there might have been dialyzable impurities.

To further clarify the cause of this absorbance we observed for the solution outside the dialysis tubing containing the δ fraction, we dialyzed the same aliquot for four days. Each day, the solution outside the tubing was removed and replaced with

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**Table 2. Effect of Dialysis in Urea/ Mercaptoethanol**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial A</th>
<th>Final A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inside</td>
<td>tubing</td>
</tr>
<tr>
<td>α + albumin</td>
<td>1.58</td>
<td>0.302</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.105</td>
<td>0.110</td>
</tr>
<tr>
<td>δ isolate</td>
<td>1.50</td>
<td>1.43</td>
</tr>
</tbody>
</table>

* For albumin and α + albumin, absorbance (A) was monitored at 440 nm. For the δ isolate, absorbance was monitored at 424 nm.

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**Table 3. Stability of δ Fraction under Various In Vitro Conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Estimated % pigment color remaining with protein or δ-fraction-derived peptide(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple chemical treatment</td>
<td></td>
</tr>
<tr>
<td>In H₂O, 37 °C, 1-4 h</td>
<td>100</td>
</tr>
<tr>
<td>In 1 mol/L HCl, 37 °C, 2-4 h</td>
<td>80-95</td>
</tr>
<tr>
<td>In 1 mol/L NaOH, 37 °C, 1-2 h</td>
<td>80-90</td>
</tr>
<tr>
<td>In boiling methanol, 65 °C, 0.5 h</td>
<td>80-95</td>
</tr>
<tr>
<td>Incubated with denaturants indicated, at 37 °C, 1 h, followed by 12-h dialysis vs H₂O</td>
<td></td>
</tr>
<tr>
<td>6 mol/L urea</td>
<td>&gt;98</td>
</tr>
<tr>
<td>4 mol/L guanidine hydrochloride</td>
<td>95</td>
</tr>
<tr>
<td>10 g/L sodium dodecyl sulfate</td>
<td>90b</td>
</tr>
<tr>
<td>10 g/L sodium deoxycholate</td>
<td>98</td>
</tr>
<tr>
<td>10 g/L Ammonyx-LO</td>
<td>98</td>
</tr>
<tr>
<td>Exposure to enzymes for 1-4 h at 37 °C and pH 7.4-8.0 followed by 12-h dialysis vs 0.05 mol/L Tris HCl buffer, pH 7.6, or elution on Sephadex G-75 column</td>
<td></td>
</tr>
<tr>
<td>Nonproteolytic enzymes:</td>
<td>95-100</td>
</tr>
<tr>
<td>β-glucuronidase,</td>
<td></td>
</tr>
<tr>
<td>β-glucosidase, lipases,</td>
<td></td>
</tr>
<tr>
<td>DNase, RNase, dextranase, hyaluronidase, creatininase, cellulase, lysozyme</td>
<td></td>
</tr>
<tr>
<td>Proteases: trypsin (4-h incubation)</td>
<td>&gt;90 on one main peptide</td>
</tr>
<tr>
<td>Pronase (4-h incubation)</td>
<td>&gt;90 on one major peptide fragment</td>
</tr>
</tbody>
</table>

* Estimates made after chemical treatments were by thin-layer chromatography; estimates made after treatments with denaturants were by spectrophotometric determinations on the dialyzates as described in the experimental section. b Complicated by marked turbidity in the incubation mixture but resolved by thin-layer chromatographic analysis. c Estimated also by gel electrofocusing.

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**Fig. 6. HPLC separation of hydrophobic azo dyes extracted from solution by Waters Sep-Pak from reaction of diazotized 2,4-dichloraniline and (a) δ fraction isolated from pathological sera; (b) bilirubin diglucuronide isolated from human bile and purified by HPLC; (c) unconjugated bilirubin (α)**
fresh buffer. The absorbance for the solutions outside the tubing steadily declined from the initial value of 0.033 to less than 0.002, while that within the tubing declined from 1.50 to 1.19 after four days. The results are consistent with there being a dialyzable impurity in the isolated δ fraction, the bulk of which is not dialyzable in urea/mercaptoethanol.

**Chemical and biological treatments to release bilirubin-like pigments from δ fraction:** Table 3 summarizes the results of our other attempts to release bilirubin-like pigments from the isolated δ fraction. Thin-layer chromatographic analysis showed that this association was resistant to treatment with acid, alkali, and boiling methanol—conditions that would have hydrolyzed simple ester (e.g., glycosidic) linkages. The less than 100% recoveries observed in some cases reflect the loss of pigment by oxidation as well as some batch-to-batch variability in the isolated δ fractions. Furthermore, none of the common protein denaturants, such as urea, guanidinium chloride, deoxycholate, or sodium dodecyl sulfate, disrupted the protein-pigment association. Ammonyx-LO was tested because it has been reported to scavenge bilirubin from all sites in serum (20), but this detergent had no visible effect on the δ fraction.

The nonproteolytic enzymes tested include a broad range of specificities for different types of covalent bonds. Their lack of effect is further strengthened by the fact that even potent and relatively nonspecific proteases such as trypsin and Pronase did not release the pigment(s) from the cleaved peptides derived from the δ fraction.

**Electrofocusing of δ bilirubin:** Figure 7 shows a typical electrofocused pattern of the δ fraction isolated from serum, referenced against those of authentic HSA, human hemoglobin, and the commercially available isoelectric pH (pI) markers run as internal standards. It is evident that, first, the major protein component(s) of the isolated δ fraction and HSA have very similar pI (4.6-4.8) and, second, this is also the region in the δ fraction (but not in HSA) that is strongly diazo-positive. Third, even after extensive proteolysis of the δ fraction, one or more of the resulting peptides with pI ≤ 4.1 remains diazo-positive.

**Discussion**

The HPLC procedure we used to separate bilirubin species in serum does not require the complete deproteinization of sera before chromatography. Instead, we used sodium sulfate solution, in a concentration suggested by the work of Yeoman (21), to precipitate primarily only proteins with relative molecular masses exceeding that of albumin. Substantially all the albumin should remain in solution for the subsequent chromatographic separation of the bilirubin species. Except for the work of Kuenzle and co-workers (2-7), in most liquid-chromatographic methods for the separation of bilirubin species in serum, the protein either is removed before the chromatographic separation (22-28) or is not resolved by the chromatographic separation (27). Thus, any bilirubin species that are very tightly associated or covalently bonded to albumin are not measured. In fact, the early method of Billing (23) has often been cited as being nonquantitative for this reason (9, 13, 22, 28).

We chromatographed serum without adding sodium sulfate to precipitate globulins and observed the same separation of four components as shown in Figure 1. Unfortunately, the HPLC column was degraded severely by these high-molecular-mass proteins, and consequently the column was shortened. With the prechromatographic separation the column is long-lived and the separation is reproducible.

The procedure given here for the prechromatography treatment of serum differs from that first used (1). Earlier, large losses of total bilirubin were observed during the precipitation step for some serum samples with high concentrations (>130 mg/L) of total bilirubin. Dilution with an equal volume of HSA (100 g/L) solution and incubation at 37°C for 20 min effectively prevented these losses. In some samples with high bilirubin concentrations, the apparent analytical recovery of α, β, and γ fractions of bilirubin, as measured by their peak areas in the HPLC separation, could be doubled by adding albumin before precipitation. Recovery of the δ fraction was affected much less, if at all. Evidently the added albumin tends to act as a carrier for bilirubin species, except for the δ fraction. This may indicate that in some very above-normal samples a substantial portion of the α, β, and γ bilirubin present can be complexed with proteins other than albumin. This is consistent with the electrophoretic studies of Fulop et al. (29).

Moreover, the HPLC separation as well as the physical and chemical tests presented here demonstrated that the association of protein with bilirubin in the δ fraction was stronger than those occurring in serum with the other bilirubin species. During the HPLC separation the known association of albumin and the other bilirubin species in solution was destroyed, as demonstrated by the elution of albumin from the column much before the α, β, and γ bilirubin fractions. The δ fraction, however, always eluted at the same time as the albumin.

The greater strength of this interaction was also evident in the procedure used to isolate the δ fraction from pathological sera. Caffeine/benzoate is known to compete with bilirubin for binding sites on albumin. In fact, caffeine/benzoate reagent readily disrupted the complexes existing in aqueous solution between albumin and the α, β, and γ fractions of bilirubin, as demonstrated by their passage through the 10 000-dalton-cutoff membrane during the isolation procedure. The δ fraction, however, remained with the proteins.

In contrast, when an unconjugated-bilirubin/albumin solution was substituted for pathological sera in the isolation procedure, we saw no evidence for bilirubin retention with the albumin, either by HPLC or by visual analysis. The only peak observed in the HPLC analysis of the retained fraction corresponded to the retention of albumin, which was also the retention of the δ fraction. The small absorbance of this sample (Figure 3) can be accounted for by the small absorbance in the yellow region of the spectrum that is characteristic of the HSA used to make up the unconjugated-bilirubin/albumin solution. This minor absorbance from HSA could not account for the yellow color in the isolated δ fraction.

The stronger interaction was also demonstrated by the results of the chemical and dialysis experiments. When the δ fraction of bilirubin was treated for relatively long times with
strong acid, strong base, or methanol, thin-layer chromatographic analysis showed that most of the yellow pigment remained attached to the proteins. With any of these treatments, simple ester bonds would have been cleaved.

The stability of the δ fraction in the presence of a strong denaturing solution was demonstrated by dialysis in urea/mercaptoethanol. Again, the complex of unconjugated bilirubin and albumin was readily broken, whereas the δ fraction remained intact. The observation of a small initial absorbance for the solution outside the dialysis tubing containing the δ fraction suggests that the δ fraction may contain some bilirubin species that is (are) less strongly attached to protein. From the HPLC analysis of the solution before dialysis it did not appear that enough of the other bilirubin species could have contaminated the δ fraction to account for the small absorbance noted. Thus, it is possible that as much as 10% of the δ fraction as isolated by the caffeine/benzoate treatment may consist of a bilirubin species that is dialyzable under these conditions. However, the observation that prolonged dialysis of this fraction led finally to a negligible increase in the absorbance for the solution outside the tubing suggests that at least 90% of the isolated material consisted of bilirubin species still attached to protein under conditions that in other work have been presented as evidence of covalent bonding (30).

The binding to albumin in the δ fraction also withstood dialysis in the presence of guanidinium chloride, deoxycholate, sodium dodecyl sulfate, and Ammonyx-LO. All but the last are known to be potent denaturing agents. Thus, an explanation for the binding of bilirubin to albumin in the δ fraction that depends upon physical adsorption or envelopment in the protein structure does not appear to be tenable.

In addition, the results of azo dye formation with dichloroaniline provided further evidence that the binding to protein in the δ fraction was especially strong. The azo dyes formed from either unconjugated or conjugated bilirubin in albumin solution were quantitatively extracted from the reaction mixture by passage through a C18 Sep-Pak; yet with the δ fraction, 50% of the total was not removed, presumably because this portion of dye was still attached to protein.

To confirm the attachment of the dyes to protein, the water-soluble dye fraction was separated from the diazo reagents by ultrafiltration and then into its components by HPLC. In this separation, even though the bilirubin species had been cleaved, that portion of dye that was not extractable by the Sep-Pak eluted with albumin at the same retention time as that for the unaltered δ fraction. This observation of an azo dye or dyes derived from bilirubin and bound to protein is consistent with an earlier report by Kanai (8).

Evidence that the isolated δ fraction did contain bilirubin in some form was obtained from the results of both the visible spectroscopy and the dioxo experiments. The spectrum of the δ fraction in the region between 350 and 550 nm was observed to be similar to that of the other bilirubin species in albumin solution (Figure 4). In addition, all fractions, even though isolated from different pathological serum pools, gave positive responses in dioxo tests, with a consistently large percentage always reacting directly. These results agree with those obtained earlier by Kuenzle et al., who found that the δ fraction was predominantly direct reacting in dioxo tests (3).

Furthermore, the HPLC separation of the extractable azo dyes formed from the reaction of the isolated fraction with dichloroaniline also demonstrated that the original δ fraction contained bilirubin residue(s). The retention times for the major components of these extractable dyes matched those for the two isomeric dyes formed from unconjugated bilirubin. Because under the same reaction conditions two different dyes are formed from the purified bilirubin diglucuronide and no evidence is found for extensive hydrolysis of these dyes during the reaction, we infer that these unconjugated azo dyes result from an unconjugated portion of the δ fraction. Further, because this portion of dye was found separated from protein, it probably was not important in the binding to protein. About 30% of the extractable dyes observed in the HPLC separation elute earlier than the two major components and thus presumably were more water soluble. They may be azo dyes of bilirubin residues conjugated with sugars including, but clearly not restricted to, glucuronic acid. This observation is consistent with the work of others who have found that although glucuronic acid may be the predominant conjugating group for bilirubin, it is not the exclusive one (31–33). Because these other dyes were observed, the isolated δ fraction cannot be a single bilirubin species bound to protein, but most likely several bound bilirubin species, of which the predominant species has half of the bilirubin moiety unconjugated. This δ isolate also contained as a major impurity essentially all the unbound albumin originally present in the serum pool from which it was isolated.

That the protein portion of the δ fraction was most probably albumin can be inferred from the isolation procedure, the HPLC separation of bilirubin species in serum, and the electrophoresis experiments. In the isolation, sodium sulfate was used at a concentration reported to precipitate, almost quantitatively, higher-molecular-mass proteins such as the globulins, but not albumin (21). Thus, in the isolation from pools of pathological sera, the only major protein that should be left after treatment is albumin. In the HPLC system developed for the separation of bilirubin species in serum, the globulins did not elute. Albumin did elute, and the retention time for its major fraction matches exactly that for the δ fraction. Finally, the observation that, by electrofocusing, the δ fraction and HSA behaved identically is the most definitive evidence we have of the nature of the protein in the δ fraction.

Although there may be noncovalent binding mechanisms as strong as the binding between protein and the bilirubin species observed in the δ fraction, the results here demonstrate a type of binding that is much stronger than the association between unconjugated bilirubin and HSA. The data are consistent with covalent binding of bilirubin species to albumin, most probably with only one dipyrrole half attached to albumin. In addition, the bilirubin moiety not attached to the protein appears to be predominantly unconjugated. Definitive proof of the protein structure and the binding site and type of the δ fraction will have to await detailed amino acid mapping of a purified fraction.

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References

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