Caffeine-Splitting of Bilirubin/Albumin Complex: Its Relevance to the Spectrophotometry of Bilirubin in Serum

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By means of gel-filtration of bilirubin/albumin mixtures, it is shown that unconjugated bilirubin remains completely linked to albumin (both human and bovine) in tetraborate buffer (pH 9.3), protein-free bilirubin appearing only when the bilirubin/albumin molar ratio exceeds two. On the other hand, bilirubin is completely set free from its protein link in the caffeine reagent. Additional chromatographic and spectrophotometric evidence is reported indicating the formation of a low-affinity complex between bilirubin and caffeine. These data explain why the spectrophotometric properties of bilirubin/albumin mixtures are matrix-dependent if measured in the tetraborate buffer but are no longer so when measured in the caffeine reagent. The relevance of these findings to the spectrophotometric "direct" assay of bilirubin in serum is discussed with reference to the occurrence of "delta-bilirubin" in pathological sera: this tightly protein-bound bilirubin fraction does not split in the presence of caffeine.

The spectral properties (absorption curve and absorption coefficients at peak wavelengths) of the bilirubin/albumin complex (BIL/ALB) vary according to the albumin source, i.e., whether human (HSA) or bovine (BSA)—when such properties are measured with the sample in tetraborate buffer (1). On the other hand, the same properties are independent of the albumin source if measurements are taken in caffeine reagent. This offers advantages for the calibration of the direct spectrophotometric assay of serum bilirubin (1). Complexing of BIL by the components of the caffeine reagent in such a way that ALB can interfere very little, if any, is the postulated mechanism for such a finding (1).

We obtained chromatographic evidence that the BIL/ALB complex is not dissociated in the tetraborate buffer (pH 9.3), but BIL is completely set free from the protein in the caffeine reagent. Moreover, spectrophotometric evidence was obtained to support the presumed formation of a low-affinity complex between BIL and caffeine.

Materials and Methods

Materials

Pure bilirubin was from Merck (cat. no. 24519); HSA (sterile solution, approx. 200 g/L) was from Istituto Sieroterapico Milanese (Milano); BSA (powder) was from Miles (Milano). Albumin calibrator (OTRH) was from Behringwerke (Marburg, F.R.G.). Bio-Gel P-10 was from Bio-Rad, Richmond, CA. All other chemicals were reagent grade. Caffeine solution was prepared as described (2), and the pH 9.3 tetraborate buffer was 0.1 mol/L plus disodium EDTA, 5 mmol/L (3).

Preparation of BIL/ALB Mixtures and of BIL Solutions

HSA and BSA were diluted or dissolved in water to give a final concentration of 40 g/L, as checked by a biuret procedure (4). From 4 to 5 mg of BIL was weighed to the nearest 0.1 mg, dissolved in 0.25 mL of dimethyl sulfoxide and 0.5 mL of 0.1 mol/L sodium carbonate solution, and diluted to 25 mL with HSA or BSA solutions. The final concentration of BIL ranged from 274 to 342 μmol/L; the final albumin concentration was about 580 μmol/L, thus ensuring a molar excess of ALB over BIL. For some experiments, BIL/ALB solutions containing a molar excess of BIL over ALB were also prepared. For the preparation of protein-free solutions, weighed amounts of BIL, dissolved in the same way, were made up to the final volume with the caffeine reagent or the tetraborate buffer. Some preparations were also supplemented with lithium chloride, final concentration 12 mmol/L, as a marker for low-molecular-mass components.

Gel-Filtration

For this we used 1.5 × 5.0 cm columns of Bio-Gel P-10, previously equilibrated with the eluent. The solution to be gel-filtered was diluted with an equal volume of eluent, and 2.0-mL aliquots were applied to the column, 1-mL fractions being collected after discarding the first 2 mL. In different experiments the eluents were either the caffeine reagent or the tetraborate buffer. In the eluted fractions the bilirubin concentration was monitored spectrophotometrically at 450 nm, the protein concentration turbidimetrically (trichloroacetic acid), and the lithium concentration by means of atomic absorption spectrometry.

Spectrophotometry

A modified caffeine reagent was prepared, its pH being increased from 7.5 to 9.1 in order to obtain better solubility of BIL. A similar solution, without caffeine, was also prepared. BIL was dissolved at the same final concentration (17 μmol/L) in these two solutions and in tetraborate buffer, and spectral scans were recorded between 400 and 500 nm, with a Varian Model DMS 90 spectrophotometer. Alternatively, caffeine was dissolved in the tetraborate buffer, to give a final concentration of 10 g/L (an almost saturated solution), and the spectrum of BIL in such solution was compared with its spectrum in tetraborate buffer.

Results

Gel-Filtration in the Tetraborate Buffer

When protein-free BIL solutions were gel-filtered, BIL was eluted as a distinct peak, after the lithium peak (Figure 1A). When BIL/HSA or BIL/BSA solutions were applied to the column, BIL was eluted together with the protein peak (Figure 2, A and B) until the BIL/ALB molar ratio did not exceed 2. At higher BIL/ALB ratios (for instance, 3.4) a second peak appeared, with the same elution volume as obtained with protein-free BIL solution (Figure 1B). No differences in the elution patterns were observed in the presence of either HSA or BSA.
Gel-Filtration in the Caffeine Reagent

When protein-free BIL solutions were gel-filtered, BIL was eluted as a distinct peak, before the lithium peak, with a lower elution volume as compared with tetraborate buffer elution (Figure 1C). When BIL/ALB (either HSA or BSA) solutions were applied to the column, BIL was completely resolved from the protein peak, before the lithium peak, with the same elution volume as obtained with protein-free BIL solution (Figure 2, C and D).

Spectrophotometric Experiments

Absorption spectra of BIL in the tetraborate buffer, in the modified caffeine reagent (pH 9.1), and in the modified reagent without caffeine are shown in Figure 3. Differential scan of BIL in the caffeine reagent, against BIL in the same reagent lacking the caffeine component, gave a peak at 482 nm. Caffeine itself does not contribute to absorbance in the 400–500 nm region, so modifications in the spectra were assumed to reflect BIL/caffeine complexing, as is the case for BIL/ALB interaction (9), the absorbance at 482 nm thereby allowing one to monitor complex formation. By increasing the caffeine concentration stepwise, at a constant BIL concentration, and plotting the absorbance values (at 482 nm) against caffeine concentration, a titration curve was obtained showing a slow, nonlinear increase in absorbance as a function of concentration, a plateau value being approached but not yet reached at a BIL/caffeine molar ratio as high as 23 · 10³. This was consistent with the formation of a low-affinity BIL/caffeine complex. Dissolving of caffeine in the tetraborate buffer induced similar modification of the absorption spectrum of BIL, and a similar titration curve was obtained by increasing the caffeine concentration.

Discussion

Results from gel-filtration experiments show that in tetraborate buffer BIL is completely linked to ALB, either HSA or BSA, until the BIL/ALB molar ratio does not exceed two. This finding was confirmed by spectrophotometric titration in our laboratory (two-wavelengths method, to be published). On the other hand, BIL is completely set free from its link with either HSA or BSA in the caffeine reagent, this being consistent with caffeine/benzoate competition with bilirubin for the binding sites on albumin (5). Of the several components of the caffeine reagent, caffeine itself appeared to be responsible for this effect, from experiments with modified reagents.
Retarded elution of BIL from the polyacrylamide gel with tetraborate buffer (more than would be accounted for by its molecular mass) may result from reversible interaction of BIL with the gel, as already described for Sephadex (6). Because this is no longer observed when the caffeine reagent is used, either caffeine is able to saturate the binding sites of the gel or a BIL/caffeine complex is formed that no longer can interact with the gel. The peculiar modifications of the BIL absorption spectrum resulting from the addition of caffeine (both to the caffeine reagent-matrix and to the tetraborate buffer), as well as the results of titration experiments, seem to indicate the formation of a low-affinity complex. BIL/caffeine interaction has been previously postulated to explain the matrix-independent spectral characteristics of BIL in caffeine solution (1) and the kinetics of diazo-coupling of BIL in the presence of caffeine (7). Our figure for the caffeine/bilirubin molar ratio that apparently is needed for complete complex formation (>23 · 10⁻⁵) is consistent with the corresponding value in the proposed method for assay of serum bilirubin based on diazo-coupling in the presence of caffeine (2).

In any case, the present results explain why the spectral properties of BIL/ALB mixtures in the caffeine reagent are the same whether HSA or BSA is used (1). A solution of caffeine in tetraborate buffer, the pH of which is better controlled than the caffeine reagent, could be an alternative diluent for the direct spectrophotometric assay of serum bilirubin. In this context, it should be considered that our data, and those previously reported by others as well (1), have been obtained with artificial mixtures of unconjugated BIL and ALB, or with animal sera enriched with unconjugated BIL. In pathological human sera containing conjugated bilirubin, a peculiar form of bilirubin occurs ("delta-bilirubin"), which is not separated from protein in the presence either of caffeine (5, 8) or of strong denaturing agents (5). This "delta" fraction can account for up to 92% of the total bilirubin in pathological sera (9), so erroneous results may be obtained in the direct spectrophotometric assay if the spectral properties of this fraction are not accurately measured and adequate calculating algorithms are not applied.

References