Interactions of Bilirubin with Bovine Serum Albumin in Aqueous Solution*

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SUMMARY

The interaction of bilirubin at concentrations less than 25 \( \mu \text{M} \) with bovine serum albumin (BSA) in aqueous solutions ranging from pH 3.5 to 8.6 was studied at room temperature by using the methods of optical rotatory dispersion (ORD) and absorption spectrophotometry between 230 and 600 nm. The binding of bilirubin by BSA at pH 5 caused a red shift of an absorption maximum from about 440 to 457 nm, the latter having a millimolar extinction coefficient of 58. This bilirubin-BSA complex exhibited probably the largest Cotton effect ever reported in the visible region, with an amplitude of approximately 1.5 \( \times 10^4 \) degrees cm\(^2\) decimole\(^{-1}\), expressed as molar rotation of bilirubin (protein concentrations were deducted), with a peak at 435 nm and a trough at 485 to 487 nm. In addition, relatively smaller anomalous rotations were observed in the region between 250 and 400 nm.

The ORD behavior of the bilirubin-BSA complex was dependent upon the pH and buffer in the system. The amplitude of the main Cotton effect decreased greatly at either side of pH 5, with significant changes in the absorption spectra. At pH 5, low concentrations of sodium acetate-acetic acid buffer (0.2 mM ionic strength) decreased the amplitude to about one-tenth of that observed in the absence of acetate buffer when the order of addition was: bilirubin, buffer, and finally excess BSA. Sodium chloride, 50 mM, added last did not affect the ORD profile at pH 5 in the presence of excess BSA; however, at pH 7.5, 0.1 mM NaCl with 10 mM Tris-HCl buffer added last reduced the Cotton effect significantly.

ORD as well as spectrophotometric titrations gave a molar ratio of unity for the bilirubin-BSA complex at both pH 5 and 7.4. At pH 5 and 25° \( \pm 2° \), the apparent association (binding) constant was estimated to be \( (5.7 \pm 1.5) \times 10^4 \text{ M}^{-1} \).

The remarkably large Cotton effects observed at pH 5 were interpreted by the formation of a dissymmetric conformation of the bilirubin molecule upon binding to BSA in a specific manner which would permit dipole–dipole coupling between the juxtaposed dipyrromethene chromophores. This conformation was considered to be modified by pH, through changes in the ionization of binding groups and by

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Interactions of bilirubin with serum albumin have been extensively studied, not only for their theoretical interest, but also because of their clinical importance (see, for example, References 1 to 6 and references cited therein). As a consequence of the latter, human serum albumin has been used in most studies. In recent years, the advances in both the instrumentation for ORD and the interpretation of rotatory results have reached a stage where this technique becomes very useful in probing the conformation of macromolecules, as well as that of small compounds in solution (for example, References 15 to 23).

Our interest in this problem is elicited from studies (24) on the bilirubin-induced swelling of mitochondria and the protective property of serum albumin. It was anticipated that the ORD technique would provide useful information about the interaction of bilirubin with bovine serum albumin. Bilirubin is usually considered to be optically inactive on the basis of the commonly accepted linear formula (Reference 1, p. 103) as shown in Scheme I. However, when bilirubin is bound to BSA, the complex shows a large Cotton effect in the visible region, as reported in our preliminary note (25). Bovine serum albumin is selected, partly because this protein is more defined than human serum albumin and a wealth of information on it is available (see, for example, References 26 to 29). The present paper communicates the details of the ORD behavior of the bilirubin-BSA complex, its association constant and the effect of pH, buffer, and salt on the
rotatory behavior of the complex, together with supporting results from spectrophotometric experiments.

EXPERIMENTAL PROCEDURE

Materials

Bilirubin—Three lots of bilirubin, reagent grade, from Mann were used in all experiments except when stated otherwise. For comparison we also used crystalline bilirubin from Sigma and crystalline bilirubin (Lot 9324) from Nutritional Biochemicals. The millimolar extinction coefficient of 60 at 453 nm in chloroform of the samples from Nutritional Biochemicals and Mann was in agreement with the “standard values” proposed. All samples in solid form were stored at about -20°C in a desiccator.

Bovine Serum Albumin—Lyophilized crystalline BSA (Lots 378-0930 and 117B-1240 from Sigma) was used without further treatment in all experiments except when stated otherwise. For comparison, crystalline preparations from Pentex, Inc., and Nutritional Biochemicals were used. Possible heterogeneity of BSA (e.g., Reference 31) and its effect on the complex with bilirubin were not investigated. Concentrations are based on direct weighing. For calculation, a molecular weight of 70,000 was used. Although this value might be considered as an upper limit of the accepted range (Reference 27; see also Reference 28), the contaminants in the samples could compensate for a slight overestimation of the molecular weight.

For solutions of BSA (see below), the extinction coefficient at 278 to 279 nm was determined at pH 7 and room temperature in the concentration range of 0.8 to 15 mg per ml. Both samples of Sigma BSA gave εₚₐₚ at 45 ± 2, Pentex, εₚₐₚ at 45 ± 0.8, and Nutritional Biochemicals, εₚₐₚ at 43.5. The εₚₐₚ value of 45 corresponds to ε₁% (absorbance of a 1% solution per cm) of 6.44. ε₁% values of 6.60 and 6.67 have been used by Tanford and Roberts (32) and by Foster and Sternberg (33), respectively.

Other Chemicals—Acetic acid, hydrochloric acid, sodium hydroxide, salts (all Baker analyzed), tris(hydroxymethyl)aminomethane (Sigma), and NaClO₄·H₂O (Fisher), in the highest purity obtainable, were purchased commercially. Deionized distilled water was redistilled in an all glass apparatus.

Methods

ORD was measured on a Cary model 60 recording spectropolarimeter, and circular dichroism on the same instrument with a Cary model 60 attachment. The slit width of the instrument was programmed for a bandwidth of 15 Å. Whenever possible, the highest sensitivity range of rotation corresponding to the full chart width of 20 millidegrees was used. The average instrumental error was about ±0.5 millidegree in the visible range under the conditions of our work. Frequent checks of the instrument and cells were made by using aqueous sucrose solutions. All samples and the reference solvents were measured separately. For ORD experiments, the optical length of cells was chosen so that the absorbance values of the solutions measured did not exceed about 0.6 between 600 and 240 nm; at 230 nm, the absorbance increased to nearly 2, and at the limit of measurements the absorbance was lower than 2.6.

The molar rotation [M] was calculated according to Equation 1

\[ [M]_a = \chi_a \cdot \text{(mol wt)}/100 = 10 \alpha/(C - d) \] (1)

where [α] and α are specific and “observed” rotations (degrees), respectively, C is the molarity of the total bilirubin in the system, and d the optical path in decimeters. No refractive index corrections were applied.

Light absorption spectra were measured on a Cary recording spectrophotometer model 11 or 14. Both instruments were calibrated by a mercury arc lamp and checked frequently by standard alkaline chromate solutions. εₚₐₚ is the extinction coefficient in units of cm⁻¹ and based on total bilirubin unless stated otherwise. pH was measured with a Beckman Zeromatic pH meter and a Metrohm (Herisau, Switzerland) Microglass electrode EA 1295U.

Preparation of Solutions—Stock solutions of bilirubin, usually about 0.2 mM, were prepared daily by dissolving in the dark about 10 mg of solid bilirubin in 10 ml of 20 mM NaOH, and diluting with distilled water to 100 ml. The solutions were stored in the dark at about 5°C. εₚₐₚ at 437 to 438 nm was 52 ± 1.1 (standard deviation from 11 experiments), measured about 30 min after preparation of the solutions, and this value remained constant for at least 6 hours. Stock solutions of BSA in distilled water ranged from 7 to 44 mg per ml and were kept at about 5°C for periods not exceeding 6 days.

Usually, a desired amount of bilirubin solution was added to a dilute protein solution; after mixing, any further component such as electrolyte or buffer was added, and the solution was then made up to a given volume. In some cases this order of addition was deliberately changed for specific reasons. The final mixture, usually at pH 6 to 7, was then adjusted to a desired pH value by addition of 0.1 N HCl or NaOH, whereby the volume of acid or base added was negligible. However, varying concentrations of additional ions resulted from these adjustments. The final mixtures were prepared close to the time of measurements and were protected from light. pH values given in this paper represent the average between the initial and final pH which did not differ by more than 0.2 unit.

RESULTS

Bilirubin in Absence of Protein—Bilirubin is sparingly soluble in neutral aqueous solutions (cf. Reference 3, p. 18; and Reference 32).

The use of both specific and molar rotation and absorptivity does not necessarily imply their independence of concentration, except for the concentration ranges indicated.

Measurements in 1.0-cm cells were completed after 1 hour. The effect of pH on the rotations of free BSA and of the bilirubin-BSA system. (a) and left ordinate, specific rotation, [a], of free BSA at 233 μm with water as the reference. ● and right ordinate, molar rotation [M] at 435 μm based on total bilirubin of bilirubin-BSA system; protein rotations obtained at the same concentration, pH, and temperature were deducted in each case from the observed over-all rotations. The system contained BSA, 3.4 to 3.7 mg per ml (49 to 53 μm); bilirubin, 21 to 24 μm; temperature, 24.5 ± 1.5°. Measurements in 0.1-cm cells were completed within about 2 hours after mixing the components. Final solutions contained varying amounts (less than 1 mm difference) of Cl⁻ and Na⁺.

When it was attempted to prepare about 20 μm bilirubin, pH 5, by acidifying a diluted stock solution, turbidity and precipitation usually occurred. Sometimes, an apparently supersaturated solution could be obtained which was stable and optically clear for a sufficient period of time so that both ORD and light absorption spectra could be measured. With 1.0 cm cells and the highest sensitivity range, no measurable rotation was detected in the range from 600 to 220 μm at 24 ± 1°, even when the solution was allowed to stand for 2 hours. Thus, for all practical purposes of this work, bilirubin might be considered optically inactive. At any rate, its molar rotations were less than about 1000 which was the sensitivity limit of the measurement. An example of the light absorption spectrum is shown in Fig. 3, Curve D; the reproducibility was poor, as expected. In most cases, at pH 5, we observed a broad maximum at about 440 μm with ε₉₀ ranging from 30 to 40 and a shoulder at 490 to 500 μm. Similar absorption behavior was obtained at pH 7.5 in the presence of 0.1 M NaClO₄ (see also References 8, 34, and 35).

At pH 7.5, using 10-cm cells, free bilirubin solutions at concentrations of about 2.5 μm did not show measurable rotation in the range from 600 to 220 μm during a period of 2 hours. When, however, the measurement was made with 25 μm bilirubin in 1.0-cm cells, the molar amplitude reached nearly 30,000 with a peak at approximately 435 μm, a trough at 410 μm, and a crossover point of 445 μm. This measurement was taken 20 min after dilution of the stock solution and the pH adjustment. After standing at room temperature for another 2 hours, the rotation increased to about 40,000 with some changes in the values of the peak, trough, and crossover point. No corresponding alteration of the light absorption spectrum was observed. On the basis of additional observations, the apparent optical activity is attributed to films of bilirubin deposited in an asymmetric array on the surfaces of the optical cell from supersaturated solutions. These artifacts, however, are not considered to affect the results obtained for systems containing bilirubin and BSA, because extrapolation to zero BSA concentration showed zero rotation (Fig. 7). Moreover, BSA apparently increased the solubility of the bile pigment in aqueous media beyond the stoichiometric value of unity (see the end of “Discussion”).

No optical activity was detected when 25 μM bilirubin in chloroform was measured in 1.0-cm cells, even when the solution was allowed to stand at room temperature for 2 hours.

BSA in Absence of Bilirubin—As shown in Fig. 2, Curve D, the ORD spectrum, from 600 to 290 μm, of BSA in water is in agreement with that reported in the literature (cf. Reference 18). Specific rotation at the 233 μm trough as a function of pH is depicted in Fig. 1. The rotation remained relatively constant between pH 4.5 and 7. Dowben and Orkin (23) have found a constant rotation at 233 μm in the range between pH 3 and 7.

The rotation at 233 μm measured 1 hour after adjusting the BSA solutions to pH 1.6, 6.1, or 9.4 was found the same as that measured after standing at room temperature for 2 hours. At pH 5, under the conditions as given for Fig. 2, no difference in the rotation at 233 μm was observed for BSA solutions containing 0.1, 1.0, or 50 mm NaCl, or sodium acetate buffer of ionic strength of 0.2, 0.2, or 20 mm. It was, therefore, not necessary to adjust the reference BSA solution to the same concentrations of Cl⁻ and Na⁺ as in the system containing BSA and bilirubin at low ionic strength.

Systems Containing Bilirubin and BSA—In Fig. 2 are shown typical ORD curves obtained at three different pH values at a molar ratio of total BSA to bilirubin of about 2.3. Under these conditions, practically complete binding of bilirubin by albumin (see below) was observed at pH 5 (see Footnote 7) and 7.5. The degree of binding at pH 3.5 was not studied. The molar and observed rotations of Fig. 2 were obtained for Curves A, B, and C by subtracting the rotations of the reference protein from the rotations of the system containing bilirubin and BSA (see “Presentation of Data” under “Discussion”). The ORD spectra of the reference BSA were measured at the same concentration and pH as for the complex. Thus, the total observed rotations for each given system can be evaluated by addition of the corresponding protein curve to Curve A, B, or C.

For easy comparison of the effect of pH on the anomalous rotations, some ORD parameters and absorption data are summarized in Table 1. In the system at pH 5, a remarkably large Cotton effect was observed; the molar amplitude from the peak at 435 μm to the trough at 485 to 487 μm was about 1.4 × 10⁶. To the best of our knowledge, this is the largest Cotton effect ever reported.
observed in the visible region. Judging from the shape of the ORD (Curve A, Fig. 2) and the light absorption spectra (Curve A, Fig. 3), the observed Cotton effect was evidently composed of multiple transitions, some of which may cause overlap with opposite signs. This explanation was substantiated by the fact that the visible absorption maximum (cf. Curve A, Fig. 3) did not coincide with the inflection point of the observed main Cotton effect, either as shown in Fig. 2 or based on the calculated ORD spectrum of the complex with a bilirubin to BSA molar ratio of unity. In addition to the main Cotton effect described, relatively smaller anomalous rotations were observed in the region of 380 to 250 nm as shown in Fig. 2.

At pH 7.5 (see Curve B, Fig. 2), the observed Cotton effect in the visible region was considerably smaller, with a trough at 497 to 500 nm, a peak at 443 to 447 nm, and a molar amplitude of about $3.5 \times 10^4$. At pH 7.5, the anomalous rotations between 210 and 320 nm were similar to those at pH 5. The difference absorption spectrum (the system containing bilirubin and BSA measured against BSA alone) in the ultraviolet region (Fig. 3) was also very similar to that obtained at pH 5.

*See Equation 1.*

| pH      | Trough position | Peak position | Molar amplitude $\times 10^4$ \(\text{peak to trough value}\) | Light absorption at visible maximum |
|---------|----------------|---------------|---------------------------------------------------------------|
| 3.5 ± 0.1 | 510             | -0.4          | 447.5 | 0.98 | 470 | 1.4 | 423 | 68.5 |
| 5.0 ± 0.05 | 460-487         | -4.6 ± 0.3a   | 439  | 9.7 ± 0.3b | 462-463 | 14.3 | 457 | 57.6 ± 1c |
| 7.5 ± 0.1 | 497-500         | -1.4 ± 0.5c   | 443-447 | 2.1 ± 0.6d | 472 | 3.5 | 471-472 | 63.5 ± 0.5d |

* Standard deviation from five experiments.

* Standard deviation from six experiments.

* Standard deviation from four experiments.
At pH 3.5 (Curve C, Fig. 2), the observed Cotton effect in the visible region was even lower than that at pH 7.5, with a trough at 510 mμ, peak at 447.5 mμ, and molar amplitude of 1.4 \times 10^5. The troughs at 510 mμ and near 385 mμ, and the peak at 447.5 mμ shifted toward longer wavelength at pH 3.5, as compared with pH 5. The light absorption at pH 3.5 (Curve C, Fig. 3) showed a pronounced maximum at 423 mμ and a shoulder at 480 to 490 mμ.

In the pH range of 8.2 to 8.6, the Cotton effects observed in systems of more acid pH were no longer discernible except for a small, broad trough at about 490 mμ. When the complex was kept for 30 min at pH 9 and then adjusted to pH 5, the molar amplitude of the main Cotton effect decreased by about 10% and the δ of the complex by 5%. This observation suggested that some irreversible change took place to a small extent at the alkaline pH. It should be pointed out that despite the large changes in the anomalous rotations between pH 4 and 7.5, the ORD change paralleled the change of the absorption spectra; the absorption maximum shifted gradually from 457 to 471 mμ.

It may be noted from Figs. 1 and 2 that on both sides of pH 5, the rotation at 435 mμ decreased markedly. On the other hand, the pH between 4 and 7.5 exerted no effect, within the limits of experimental error, on the rotation at 233 mμ of BSA both in the presence or absence of bilirubin (cf. Fig. 1). At pH 8.4, [δ]m based on BSA was −9420 for the complex as compared to −7980 in the absence of bilirubin under the conditions as detailed in Fig. 2.

**Effect of Time and Temperature**—In the systems containing excess of BSA over bilirubin in the pH range of 5 to 7.5, the rotations and absorption in the visible range, measured about 150 min after mixing, did not decrease by more than 5% as compared with the values measured after about 25 min. This was not true for the systems at lower pH; a decrease of about 60% was observed at pH 3.7. This type of decrease, in general, was more serious when bilirubin was in excess. No significant effect of temperature on the rotation and absorption of the complex was observed between 20 and 30° at pH 5.

**Variation of Sources of BSA and Bilirubin**—Bilirubin and BSA from different commercial sources were compared at both pH 5 and 7.5 under the same conditions as those given for Fig. 2. No difference larger than the experimental error was observed among the various samples of bilirubin with respect to ORD and absorption spectral data.

Some anomalies were, however, noticed for BSA. When BSA from Pentex was used at pH 5, the complex showed a molar amplitude of the observed Cotton effect in the visible region about 20% lower than that of the complex with BSA from Sigma. The BSA from Nutritional Biochemicals gave results practically identical with those of the Sigma sample at pH 5. At pH 7.8, however, were observed a decrease of about 30% in the amplitude, a red shift of 10 mμ of the main Cotton effect, and a blue shift of about 10 mμ of the trough. The absorption spectra of the complex with Nutritional Biochemicals BSA did not differ from those with Sigma BSA at either pH 5 or 7.6.

**Effect of Buffer and Salts**—The Cotton effects and light absorption of the bilirubin-BSA complex were affected not only by buffer and salts, but also by the order of the addition of the reagents, as shown in Table II. For example, at an ionic strength of 0.2 mM of sodium acetate-acetic acid "buffer" at pH 5, and order of addition in the sequence bilirubin, acetate buffer, and finally BSA, the amplitude of the observed Cotton effect was only about one-tenth (Curve B, Fig. 4) of that obtained in the absence of the buffer (Curve A, Fig. 2). Upon standing, the rotations increased slightly (cf. Table II). If, however, the order of addition was BSA, then acetate buffer, and finally bilirubin, the amplitude (Curve A, Fig. 4) remained constant for at least 2 hours and was the same as that in the absence of acetate buffer (Curve A, Fig. 2). Analogous phenomena were also observed in the light absorption spectrum (Fig. 5 and Table II).

At higher ionic strength of acetate buffer (20 mM), the amplitude of the Cotton effect in the visible range measured within 1 hour after preparation was only about one-fifth of the maximum obtainable (see Table II), even when the order of addition was BSA, then acetate buffer, and finally bilirubin. Other effects are also evident from Table II. Apart from their intrinsic interest, all these results obtained with acetate did not permit the practical use of high concentration of buffer near pH 5.

Unlike sodium acetate buffer, 0.6 mM sodium chloride, added to the bilirubin solution before the protein under the condition described in Fig. 2 had no influence on either the Cotton effects or the light absorption. Likewise, 50 mM NaCl added last to the system did not cause significant changes with time and showed the same behavior as the system in the absence of NaCl. However, at lower BSA concentrations, irregularities were observed in the presence of NaCl.

In order to keep constant ionic strength in the system and to suppress electrostatic effects, an electrolyte with magnitude of concentration of 0.1 M should usually be added. However, in view of the complications described, it was preferred to work...
FIG. 4. Effect of the order of addition of acetate buffer on the main Cotton effects of bilirubin in the presence of BSA. From total rotations were deducted the protein rotations obtained at the same pH, temperature, and concentration of BSA and buffer; pH 5.0 ± 0.05; temperature, 23.2° ± 0.2°. Order of addition of solutions and final concentrations for Curve A are: BSA, 3.6 mg per ml (51 μM); sodium acetate-acetic acid buffer, ionic strength 0.2 mM; and bilirubin, 22 μM. Order of addition of solutions for Curve B is: bilirubin, acetate buffer, and BSA; the same final concentrations as for Curve A. Both systems also contained additional 0.26 mM Na⁺ and 0.5 mM Cl⁻. Both measurements in 0.1-cm cells were started 20 min after mixing all components.

At low ionic strength, at least at pH 5 where close agreement between rotation values was obtained in the presence and absence of added salt under certain conditions. Except at very low concentrations, the isoinionic point of BSA is near pH 5 (28) so that the average net charge on the protein should be small despite some ion binding at this pH. When a sample of Sigma BSA was exhaustively dialyzed at 4° against distilled water and then used, its complex with bilirubin (conditions as for Curve A, Fig. 2) showed practically the same rotation and absorption in the visible range as the untreated sample (Figs. 2 and 3). At pH 7.4, however, large salt effects on the anomalous rotations were encountered which are also evident from the data of Fig. 7 (see below). Higher concentrations of BSA (>30 μM), 0.1 M NaCl with 10 mM ionic strength of Tris-HCl buffer added last, decreased the rotations in the visible range to about one-half of the value obtained at low ionic strength (0.8 mM). The light absorption, however, was similar in both cases.

Titrations of Bilirubin by BSA and Vice Versa—Figs. 6 and 7 depict the results of ORD titrations in which the bilirubin concentration was kept constant. The amplitude of the main Cotton effect was used as a measure of the change. Because of the broadness of both peaks and troughs, practically the same results were obtained by taking either the amplitudes of the slightly shifted Cotton effects or the rotations at constant wave lengths. In view of the effect of buffer and salt on the rotations as described, the titrations at pH 5 were carried out in the absence of buffer.

In the presence of constant bilirubin concentration (Fig. 6), an increase in BSA increased the amplitude of the Cotton effect. Not until a molar ratio of 1 between bilirubin and BSA was approached did the curve level off. A close correspondence with this titration curve was obtained when the light absorption was used as a measure instead of the ORD amplitude (Fig. 6).

A titration at constant BSA concentration at pH 5 by bilirubin has been reported in our preliminary note (25). Assuming reversibility, these data were amenable to analysis (36) as shown in Equation 2

\[ \frac{1}{r} = \frac{1}{nK(A)} + \frac{1}{n} \]

where \( r \) is the ratio of moles of bound bilirubin to total moles of protein; \( A \), concentration of bilirubin not bound in the complex; \( n \), average number of moles of bilirubin per mole of protein in the complex; and, \( K \), association constant.

A plot of \( 1/r \) versus \( 1/(A) \) is presented in Fig. 8. The concentration of bound bilirubin was evaluated from the fraction of the maximal amplitude of the main Cotton effect obtainable. Extrapolation of amplitude versus \( 1/[\text{BSA}] \) for the data of Fig. 6 gave \( \Delta \alpha = 3.4 \) degrees per dm, or \( [M] = 1.5 \times 10^6 \), which is in good agreement with the value calculated from the initial slope in the titration as previously reported (25). From the reciprocal slope (Fig. 8), an association constant, \( K = (5.7 \pm 1.5) \times 10^6 \text{ M}^{-1} \), was obtained at pH 5 and 25°. The intercept is close to 1, verifying a molar ratio of 1 for bilirubin to BSA in the complex.
**Fig. 6.** ORD and spectrophotometric titrations of bilirubin by BSA at pH 5.0 ± 0.5. Abscissa, ratio of total moles of BSA to total moles of bilirubin (BR) in the system. ▲ and right ordinate, absorbance per cm at 457 m. ○ and left ordinate, amplitude of the main Cotton effect in degrees per dm for the systems containing bilirubin in the presence of BSA (BSA rotations deducted). Actual ORD measurements were conducted in cells of 0.1-cm optical path; bilirubin, 23 μM; temperature (ORD) 24.5 ± 0.5°; (absorption spectra) 25° ± 1°; rotation and absorption measurements were completed within about 30 and 35 min, respectively, after mixing the components. References for ORD and absorption contained BSA at the same concentration and pH as the experimental.

**Fig. 7.** ORD and spectrophotometric titrations of bilirubin (BR) by BSA in the presence of NaCl at pH 7.4 ± 0.1. Abscissa, ratio of total moles of BSA to total moles of bilirubin in the system. ▲ and right ordinate, absorbance per cm at 470 m. ○ and left ordinate, amplitude (BSA deducted) of the main Cotton effect in degrees per dm. Actual ORD measurements were conducted in cells of 0.1-cm optical path; bilirubin, 24 μM; NaCl, 0.1 M; Tris-HCl, ionic strength, 10 mM; temperature (OR) 24.5 ± 0.5°; (absorption spectra) 27° ± 1°. Rotation and absorption measurements were completed within about 80 min after mixing the components. References for both ORD and absorption contained BSA at the same concentration and pH as the experimental. Note the nonparallelism between the rotation and absorption data at high ratios of BSA to bilirubin.

At pH 7.4, data obtained in Tris-HClO4 buffer at relatively low and constant BSA concentration (1.7 μM) could not be extended to higher bilirubin concentrations. Nevertheless, a molar ratio of unity in the complex was again obtained. Although a K value was not quantitatively evaluated, it should be at least one order of magnitude higher than that obtained at pH 5. A number of titration curves obtained from five separate experiments gave essentially the same results. When the titration at pH 7.4 was conducted at a constant bilirubin concentration, some peculiarities as shown in Fig. 7 were observed. It nevertheless was possible to deduce again a molar ratio of 1 which was also indicated by the spectral data of Fig. 7.

**DISCUSSION**

**Presentation of Data**—In our presentation of ORD data, we subtract the rotations of BSA from the total rotations of bilirubin in the presence of BSA (the bilirubin-BSA complex) measured under the same conditions. This mode of presentation is based on the assumption that bilirubin does not affect the rotation of BSA. It appears to be the only practical way to present our results meaningfully. Neither the use of systems containing only stoichiometric amounts of bilirubin and BSA (i.e. in the molar ratio of unity) nor the actual isolation of the complex would meet the requirements of our experiments. Dilute concentrations of the order of 20 μM bilirubin are usually used because of the intolerance of high absorbance by the spectropolarimeter. Under such conditions, dissociations up to nearly 10% would occur if the pure complex were used at pH 5.

On the other hand, we are not unaware of the possibility that the BSA may be perturbed by bilirubin in the complex resulting in local conformational changes in the protein. Nevertheless, this kind of perturbation is not evident from the rotation of the complex at 233 μM, which remains practically the same as that of the free BSA (see below). It is, however, possible that compensations of the rotations caused by other transitions in the complex may be the reason for the similar behavior of the free BSA and the complex observed in the far ultraviolet region.

**ORD of Bilirubin-BSA Complex at pH 5**—The molar amplitude of the observed Cotton effect in the visible region for the bilirubin-BSA complex at pH 5 amounts to about 1.5 × 10⁷ which is even larger than that observed for resolved hexahelicene. Newman, Darlak, and Tsai (37) have reported that hexahelicene in methanol exhibits a molar amplitude of 1.2 × 10⁷ in the region of 232 to 270 mμ (refractive index apparently not corrected). The amplitudes of the Cotton effect for the fluorescein-BSA complex (23), for the protohematin-polysyaline complex (38) and for the...
complexes of hematin with several lysine-rich proteins for example, are several times smaller than that measured for the bilirubin-BSA complex. Among the natural tetrapyrrol-linked proteins studied, cytochrome oxidase has the largest Soret-Cotton effect, but the molar amplitude per hemec is about 300,000 (39).

As in the case of hexahelices, the extremely large Cotton effect of the bilirubin-BSA complex at pH 5 is very likely associated with a high degree of inherent dissymmetry (see Reference 40) and dipole-dipole coupling (41) in the bound bilirubin molecule. It can be shown by space-filling molecular models that by rotation around the C—C single bonds of bilirubin connecting the two pairs of conjugated pyrrole rings, numerous conformations of varying degrees of dissymmetry can be produced (for example, Fig. 9). In some of these helical conformations, dipole-dipole coupling between the dipyrrylmethene chromophores of the bilirubin may be possible by their juxtaposition. A likely and relevant conformation as shown in Fig. 9 (left) could be formed at pH 5 by specific and noncovalent interactions of bilirubin attached to a certain site of the BSA molecule. Intramolecular hydrogen bonding involving carboxyl, carbonyl, and nitrogenous groups of pyrrole may further stabilize a given conformation (42), especially if the “local” medium around the bound bilirubin is less polar. Moscowsitz et al. (40) have suggested that because conformations are stabilized by such kinds of hydrogen bonds, even free urobilins in chloroform show large Cotton effects (40, 43). In contrast, free bilirubin either in chloroform or in water at pH 5 is optically inactive, or its rotation is below the limits of detection.

A variety of noncovalent interactions may be involved in the binding of bilirubin by BSA, such as hydrogen bonding or electrostatic interactions through the bilirubin carboxyl and carbonyl groups, nitrogenous and other functional groups with the protein, the bilirubin pyrrole nitrogens, etc. In addition, hydrophobic interactions (including dispersion forces) involving the bilirubin side chains and nonpolar groups of BSA may contribute to the stability of the complex. Possible effects of aggregation of BSA (see Reference 26) are not considered, since the molar amplitude of the complex was independent of BSA concentration over a wide range.

Our proposed structure of the bilirubin bound to BSA possesses, in a way, the sense of a right handed helix. From very simple considerations, one might expect the Cotton effect to be positive for this helical sense (cf. References 44 and 45). On the other hand, the observed main Cotton effect is apparently negative. However, it must be pointed out that the observed Cotton effect is composed of multiple transitions and indeed also consists of an evidently complicated couplet.

Effect of pH—The light absorption data between pH 5 and 8.6, in systems with excess BSA, both in the presence (Fig. 7) and the absence of salt (Fig. 3), suggest the formation of the bilirubin-BSA complex in different forms with no indication of significant dissociation of bilirubin. A small shoulder in the light absorption spectrum near 440 nm at pH 5 is not attributed to free bilirubin, because the spectrum remains practically unchanged in this region even in the presence of a large excess of BSA (Fig. 6). The decrease of the main Cotton effects on both sides of pH 5 (Fig. 1) is therefore not to be taken as a measure of the dissociation of the complex, at least not in the pH range of 5 to 8.6.

The gradual changes in both absorption and rotatory properties with pH in the range of 5 to 7.5 are most likely related to conformational changes of the bilirubin molecule bound at specific sites. Changes in the state of ionization in the pH range between 5 and 9 of various ionizable groups of either BSA (26) or bilirubin (pyrrole nitrogens, propionic acid carboxyl groups, etc.) may change the mode of interaction between the complex components. It appears that less dissymmetric conformations with reduced degrees of dipole-dipole coupling between the chromophores of the two bilirubin halves, separated by the center methylene group, are formed as the pH increases from 5 to 8 (Fig. 9, middle and right). Some pH-dependent, conformational changes of the protein may also affect both the mode of binding and the extrinsic influence of the dissymmetric protein environment on the bound bilirubin molecule. These effects may become more prominent at relatively low rotations. Preliminary measurements of circular dichroism in the visible region showed both negative (longer wave lengths) and positive bands at pH 5, and only a negative band at pH 7.5. Further work on dichroism is in progress.

At more extreme pH values, BSA is known to undergo con-
formational transitions (26, 48-48) and aggregation in the presence of salts (29). Thus the intramolecular changes of BSA may cause, at least in part, the observed differences in the interaction between bilirubin and BSA at these extreme pH values as shown by the ORD and light absorption results.

At pH 3.5, the rotation at 233 nm is slightly more negative for the complex than for the free BSA; at pH 8.4, the complex shows even more negative rotation than the free BSA at pH 5. Thus the effect is a change upon complex formation at these pH values (cf. Reference 20, p. 89). Evidence is available for the stabilization of BSA by anions (28). At pH values between pH 5 and 7.5, any difference at 233 nm may be either insignificant or compensated by other rotations.

**Effect of Buffer and Salts**—At least in systems with acetate buffer of low ionic strength added to the bilirubin first, the slow formation of complex cannot reasonably be attributed to an interaction between the buffer and BSA, since no effect is observed when the buffer is added to BSA first. It appears that the buffer forms some complex with bilirubin which dissolves slowly in the presence of BSA and subsequently forms the bilirubin-BSA complex. This complex of buffer and bilirubin may involve combined electrostatic and hydrophobic interactions of the acetate ion with both some positively charged regions and nonpolar moieties of the bilirubin molecule. Obviously, these latter interactions do not exist with chloride ions. At higher ionic strength of buffer (20 mm) the results again indicate nonequilibrium conditions, but in addition, interactions between acetate and BSA also apparently become important.

Although chloride ions are known to bind to BSA (e.g., Reference 29), this interaction apparently has little effect on the bilirubin-BSA complex at pH 5. It is therefore surprising that NaCl has a large effect on rotations of the complex at pH 7.4 in the visible range, even when added as the last component, since little chloride binding should occur at this pH (see Reference 29, and earlier references cited therein). Moreover, the absorption spectrum remains unaffected. These findings demonstrate the high sensitivity of the ORD of the complex at pH 7.4 to changes in the ionic environment.

**Titrations**—ORD and light absorption titration data give a bilirubin to BSA molar ratio of unity for the complex at both pH 5 and 7.4. However, Barac (11) has reported a ratio of about 2.4. At pH 7.5, a ratio of 1 has been found (24) for the BSA protection of uncoupling of oxidative phosphorylation by bilirubin and of the energy-required mitochondrial swelling induced by the bile pigment (see also Reference 6 and Footnote 8). A molar ratio of about 2 has been reported by various investigators for the complex of bilirubin with human serum albumin near physiological pH values (5, 6, 8-10, 13). However, ratios of about 3 (49) and 20 (50) have also been claimed. Apparently, the molar ratio of the complex components depends on the method and conditions used for its determination.

We have observed that in the systems studied, the excess bilirubin over the stochiometric amount of BSA does not precipitate. Therefore, the estimated "free" bilirubin concentrations used in calculation of the apparent association constant may include that part of the bilirubin which reacts in a nonspecific manner with BSA whereby both absorption spectra and rotational properties of the free bilirubin remain unchanged. With consideration of such nonspecific and apparently weaker binding, the computed association constant would constitute a lower limit.

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