# OPTICAL ROTATORY DISPERSION OF BILIRUBIN BOUND TO BOVINE SERUM ALBUMIN

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From chemical, biochemical and clinical viewpoints, studies of the binding of bilirubin by protein, in particular serum albumin, are of considerable importance (e.g. 1-5). Our interest in this problem is elicited from the observation (6) on the bilirubin-induced mitochondrial swelling and the protective property of bovine serum albumin (BSA). Recently, we have found that when bilirubin is bound to BSA in aqueous solution, extremely large Cotton effects are developed in the region 400-500 mμ. These findings afford a new and independent means to probe various facets of interaction between bilirubin and the protein.

Experimental -- Bilirubin, reagent grade, was obtained from Mann Research Lab., and crystalline BSA, from Sigma Chemical Co. Stock solutions of bilirubin prepared daily in 2 mM aqueous NaOH were protected from light and stored at 0-5°. Light-absorption spectra were recorded on a Cary Model 11 spectrophotometer. Optical rotatory dispersion (ORD) was measured with a Cary Model 60 spectropolarimeter. The maximal absorbance of the solutions was less than 0.6 in the visible region; only at 230 mμ did it increase to about 2. The slit width of the instrument was pro-

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grammed for a band width of 15 Å. $[M]_\lambda = [\alpha]_\lambda \cdot (\text{Mol.wt.})/100 = 10\alpha/(C \cdot d)$ where $[M]_\lambda$, $[\alpha]_\lambda$ and $\alpha$ are molar, specific and observed rotations, respectively; C is the molarity of bilirubin and d the optical path in dm.

Results and Discussion -- In our presentation*, we subtract the rotations of BSA alone from the total rotations of bilirubin in the presence of BSA (the bilirubin-BSA complex), measured under identical conditions. Examples of ORD profiles of the bilirubin at an excess of BSA at two differ-

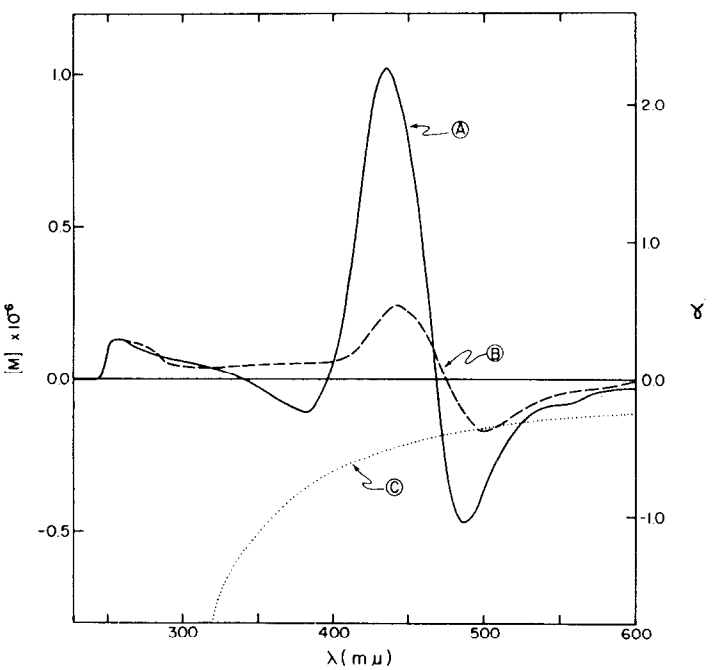


Fig. 1. ORD of bilirubin in the presence of BSA

The left ordinate is the molar rotation, $[M]$, (see text), and relates to Curves A and B. The right ordinate is the "observed" rotation in degrees per dm for the systems described (Curves A, B and C). The protein rotations are deducted for Curves A and B. Measurements at 23° were completed within approximately 90 minutes after mixing the components. Curve A: bilirubin, 22.3 μM; BSA, 3.59 mg/ml; $Cl^-$, 0.5 mM; $Na^+$, 0.26 mM; pH 5.0 ± 0.05. Curve B: same as A, except $Cl^-$, 0.05 mM; $Na^+$, 0.76 mM; pH 7.5 ± 0.1. Curve C: BSA, 3.59 mg/ml; pH 5.0; a practically identical spectrum of BSA is obtained at pH 7.5.

*This mode of presentation is based on the assumption that bilirubin does not affect the rotation of BSA. However, the validity of this assumption remains to be shown (see below, rotations at 233 mμ).

ent pH values are depicted in Fig. 1 (Curves A and B). The well-known ORD spectrum of BSA (Fig. 1, Curve C) is typical for a protein. Apparently, free bilirubin is optically inactive while the bilirubin-BSA complex shows large anomalous rotations in the range 400-500 mμ. For the system at pH 5 (Curve A, Fig. 1), the overall molar amplitude of the main Cotton effects is approximately 1.5 million. The curve clearly indicates that the anomalous rotations consist of multiple Cotton effects, some of which most likely are of opposite sign. The apparent inflection point does not coincide with the light-absorption maximum at 457 mμ (Curve A, Fig. 2). This discrepancy may well be attributed to the existence of multiple transitions. Indeed, the light-absorption spectrum is not symmetrical and exhibits a shoulder near 440 mμ. Cotton effects are also observed in the region between 250-400 mμ but their magnitude is smaller than those observed at the higher wavelengths. Again judging from their shape, these anomalous rotations are probably due to multiple Cotton effects. Small and broad maxima are also discernable in the ultraviolet absorption spectrum as shown in Fig. 2.

The ORD behavior is affected by a number of factors, such as the pH, type of ions present, ionic strength, and, with some added ions, by the order of addition of the components. When the order of mixing was bilirubin, sodium acetate buffer (ionic strength 0.2 mM) and finally BSA (conditions as given for Fig. 1), the amplitude of the main Cotton effects in the region 400-500 mμ at pH 5 decreased to about one-tenth of that observed in the absence of acetate. Under the same conditions, 0.6 mM NaCl did not affect the amplitude. Also, if the acetate was added to the BSA solution and then bilirubin, the amplitude remained practically unaffected. The effect of ions

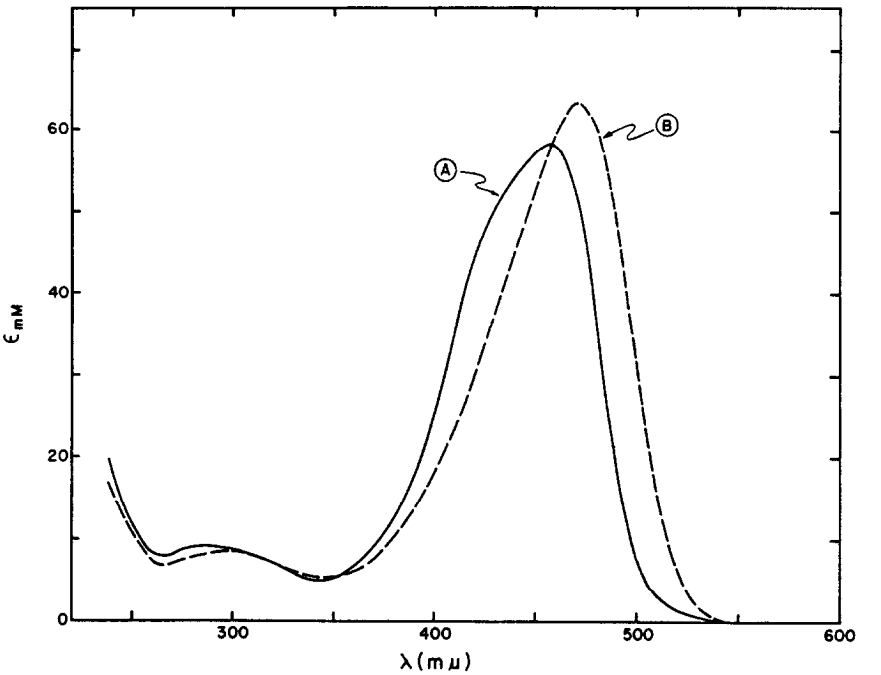


Fig. 2. Light-absorption spectra of bilirubin in the presence of BSA

The ordinate is the millimolar extinction coefficient per cm based on bilirubin. Measurements at 26° were made against the reference system containing BSA at the same concentration and pH; these were completed within approximately 45 minutes after mixing the components. Curve A: bilirubin, 22.5 μM; BSA, 3.58 mg/ml; $Cl^-$, 0.7 mM; $Na^+$, 0.26 mM; pH 5.0 ± 0.1. Curve B: same as A, except no $Cl^-$; $Na^+$, 0.56 mM; pH 7.7 ± 0.1. For free bilirubin an absorption maximum is found at about 440 mμ (cf. 3) with $\epsilon_{mM}$ of 30-40 in freshly-prepared supersaturated solutions at either pH 5.0 or 7.4. A shoulder at 490-500 mμ is also observed at both pH values.

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at pH 7.4 was different. Details will be reported elsewhere.

In a plot (not shown) of the amplitude of the Cotton effects in the region 400-500 mμ as a function of pH, a relatively broad maximum is found at pH 4.6-5.2. The magnitude of the anomalous rotations at pH 7.5 (Curve B, Fig. 1) is much smaller than that at pH 5. The light-absorption maximum is at 471 mμ (see Curve B, Fig. 2, which applies also to pH 7.5).

Optical rotatory titrations were performed by varying the concentration

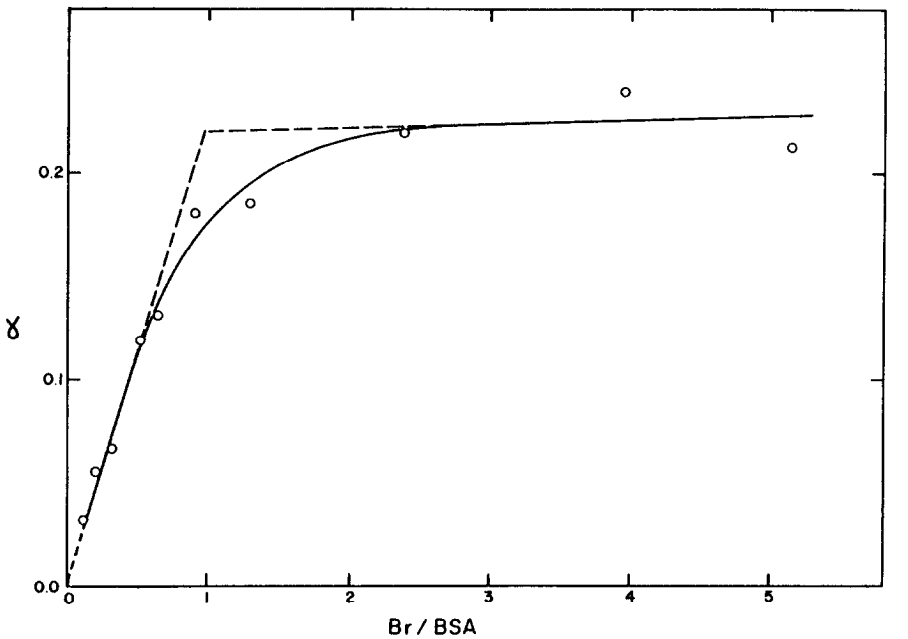


Fig. 3. ORD titration of BSA with bilirubin

The ordinate is the amplitude of the main Cotton effect (range 400-500 mμ) in degrees per dm; the rotations of BSA are deducted. The abscissa is the molar ratio of bilirubin to the BSA present in the system. pH 5.0 ± 0.2; BSA, 0.12 mg/ml. The molecular weight of BSA is assumed to be 70,000. Measurements at 23° were completed within approximately 60 minutes after mixing the components.

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of either bilirubin or BSA while keeping the other constant. Figure 3 shows, as an example, the system at pH 5. Extrapolation of the curve gives, at saturation, a molar ratio (bilirubin to BSA) close to unity. This ratio is further verified by a more rigorous analysis (7). Assuming reversibility, the apparent association constant at pH 5 (near the isoionic point of BSA) is estimated to be of the order of $10^6$ $M^{-1}$. Similarly, a molar ratio close to 1 is indicated for the complex at pH 7.4 at higher ionic strength. The same value was found in the BSA protection of the energy-required mitochondrial swelling induced by bilirubin (6).

The magnitude of rotation of BSA at the trough at 233 mμ remained practically constant between pH 4.5 and 7.5 (see also ref. 8), both in the presence and absence of bilirubin. However, the ORD profile of the BSA-bilirubin complex is strongly pH-dependent. The unusually large Cotton effects of this complex at pH 5 are likely to be due to the formation of twisted dipyrrylmethene chromophores of fixed chirality (not unlike the urobilins, ref. 9) with possible dipole-dipole coupling between them. Helical conformations of this kind may be formed by non-covalent and specific interactions between BSA and bilirubin. In addition, various hydrogen bonds may thus be formed within the bilirubin molecule itself. It is then suggested that on change of pH the observed changes of optical rotation and light absorption reflect, at least in part, conformational changes in the bound bilirubin molecule which may result from changes with pH of the state of ionization of groups participating in the binding. These conformations exhibit different degrees of dissymmetry and dipole coupling. In addition to this internal dissymmetry, possible contributions to the rotation due to interaction between bilirubin and the asymmetric environment of the protein in the region of the attachment of bilirubin should also be considered (cf. ref. 8).

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## References

1. Martin, N. H., J. Am. Chem. Soc., 71, 1230 (1949).
2. Klatskin, G., and Bungards, L., J. Clin. Invest., 35, 537 (1956).
3. Odell, G. B., J. Clin. Invest., 38, 823 (1959); J. Pediat., 55, 268 (1959); ibid., 68, 164 (1966).

4. Arias, I. M., in H. Popper and F. Schaffner (Eds.), Progr. Liver Diseases, Vol. 1, Grune and Stratton, New York, 1961, p. 187.
5. Schmid, R., Diamond, I., Hammaker, L., and Gundersen, C. B., Nature, 206, 1041 (1965).
6. Mustafa, M. G., Cowger, M. L., and King. T. E., Biochem. Biophys. Res. Commun., 29, 661 (1967).
7. Klotz, I. M., in H. Neurath and K. Bailey (Eds.), The Proteins, Vol. 1B, Academic Press, New York, 1953, p. 727.
8. Dowben, R. M., and Orkin, S. H., Proc. Natl. Acad. Sci. U. S., 58, 2051 (1967).
9. Moscowitz, A., Krueger, W. C., Kay, I. T., Skewes, S., and Bruckenstein, S., Proc. Natl. Acad. Sci. U. S., 52, 1190 (1964).