THE EFFECT OF LOWER ALIPHATIC ALCOHOLS ON THE CIRCULAR DICHROISM OF THE COMPLEX BILIRUBIN–HUMAN SERUM ALBUMIN IN AQUEOUS SOLUTION

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Received 17 January 1974

1. Introduction

Previous investigations on the optical properties (CD* and light absorption) of the bilirubin–human serum albumin complex in aqueous solution revealed the existence of large and proximate CD bands of opposite sign between 400 and 500 nm, which showed a pH-dependent and reversible inversion in their sign occurring near pH 5 [1,2] (see also [3]). The addition of electrolytes at pH 5 also caused a simultaneous inversion in the sign of both observed CD bands, with some shifts in the position of their extrema [4,5]. The pH-dependent inversion of the CD bands has recently also been observed in human adult blood serum [6].

The present report demonstrates effects of short-chain aliphatic alcohols on the visible-range CD spectra of the bilirubin–HSA complex. It is shown that even at neutral pH, relatively low concentrations of 1-propanol and some higher alcohols affect the CD spectra markedly and may cause inversion in the sign of the observed bands. In contrast, the corresponding light-absorption spectra do not change significantly upon addition of the alcohol. Also, 2-propanol does not show the inversion effects even at higher concentrations. The effects observed in the CD at relatively low concentrations of the alcohols suggest specific interactions with the complex components, rather than medium effects, leading to significant changes in the conformation and hence in the electric transition dipole coupling in the bound bilirubin.

* *Abbreviations:* CD, circular dichroism; HSA, human serum albumin; BSA, bovine serum albumin.

2. Materials and methods

Bilirubin was obtained from Sigma. It showed only traces of impurities (isomers of bilirubin) on thin-layer chromatography [7]. Human serum albumin, crystallized, was obtained from Pentex and treated with charcoal according to Chen [8]. A molecular weight of 68 000 was assumed. 1-Propanol (Riedel–de Haën) was dried over $CaSO_4$ and distilled ($n_D^{20} = 1.3850$). Other substances used were of analytical grade.

CD measurements were carried out on a Cary Model 60 recording spectropolarimeter with a Model 6002 accessory. The optical density of the solutions measured did not exceed about 1.2. For other experimental details and preparation of the complexes, see ref. [2]. The alcohols were always added as the last component of the mixture. Their concentrations in aqueous solution are expressed in volume percent.

3. Results and discussion

The effect of increasing the concentration of 1-propanol on the CD spectrum of the bilirubin–HSA complex in aqueous solution is shown in fig. 1. At 2.5% (v/v) 1-propanol, both observed

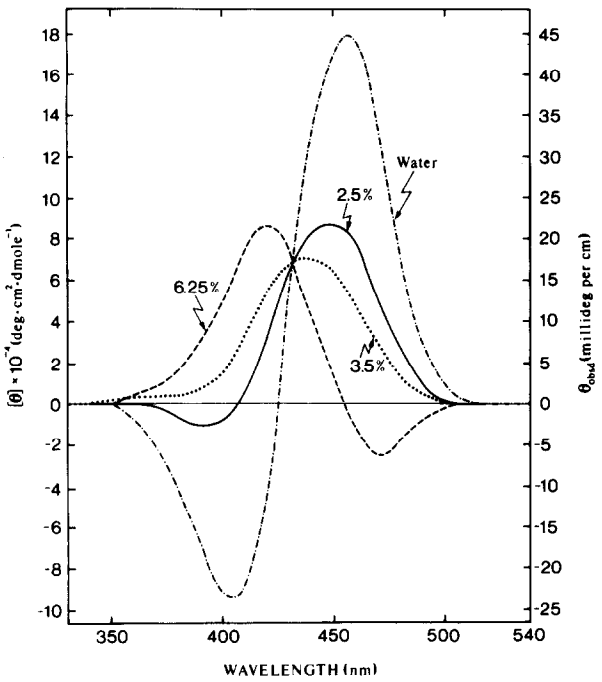

Fig. 1. CD spectra of the bilirubin–HSA complex in aqueous solution and in the presence of different concentrations of 1-propanol. Bilirubin, 2.5×10^{-5} M; HSA, 5×10^{-5} M; NaCl, 0.1 M; Tris–HCl, 0.02 M; pH 7.4; temp., 27.0 ± 0.5°C. The numbers designating the curves are the volume percent of 1-propanol present in the solution. Measurements were started about 20 min after preparation of the solutions. Reference solvents include all components, except bilirubin. Molar ellipticities [θ] are based on the total bilirubin concentration.

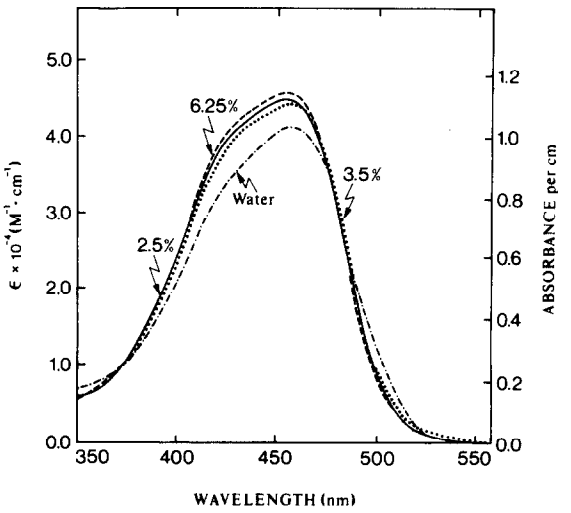

Fig. 2. Light-absorption spectra of the bilirubin–HSA systems described in fig. 1. Temp., 24 ± 1°C. Measurements were started within about 30 min from preparation of the solutions. Molar extinction coefficients ϵ are based on the total bilirubin concentration.

visible-range CD bands, which are of opposite sign, decrease in magnitude, with concomitant shifts of the extrema to shorter wavelengths. In the presence of 3.5% (v/v) 1-propanol, only one positive CD band is observed, while with 6.25% 1-propanol (0.83 M) included, both observed bands are of opposite sign compared with the aqueous solution in the absence of propanol. The longer wavelength band is much smaller in the presence of 1-propanol and both observed band extrema appear to be shifted by about 15 nm to longer wavelengths. An isoelliptic point for all spectra is observed near 432 nm. Increasing the concentration of HSA to 2.1×10^{-4} M causes an increase of about 20% in the ellipticity values of the band extrema. This may indicate, under the conditions given for figs. 1 and 2, the presence of some free bilirubin, which is not expected to contribute significantly to the observed ellipticities [2]. In contrast to the large variations of the CD spectra with the concentration of 1-propanol, the corresponding light-absorption spectra do not differ largely (fig. 2).

Dilution (at constant ionic strength) of a solution of the complex containing 6.25% 1-propanol to a solution containing 2.5% 1-propanol results practically in the same inverted CD spectrum as obtained for a solution prepared directly to the same composition. This (molar) spectrum is also similar to that shown in fig. 1 for 2.5% 1-propanol. Thus, reversibility is indicated with regard to the effect of 1-propanol.

CD measurements of the bilirubin complex, carried out in the presence of various concentrations of 1-propanol (conditions as given for fig. 1) with HSA monomer separated on a G-200 Sephadex column, gave practically the same results as obtained in the presence of the unfractionated albumin.

30% Methanol (conditions as given for fig. 1) caused an increase of about 50% in the ellipticity values of the band extrema without changes in their sign. In 10% ethanol, the magnitude of both band extrema diminished. Similar observations were made up to 15% 2-propanol. It therefore appears that both the hydrocarbon chain length and the position of the

hydroxyl group have an important effect on the protein complex. This is also demonstrated by the higher efficiency of longer-chain alcohols in causing changes in the sign of the CD bands: both 3% 1-butanol and 0.5% 1-hexanol were sufficient to effect these inversions.

A sample of human adult serum was diluted with water (final albumin concentration, about 5×10^{-5} M). Bilirubin was then added up to 2.5×10^{-5} M and 1-propanol to 6.3%. The CD spectrum obtained was similar to that of the corresponding complex shown in fig. 1. The similarity between the CD spectra of undiluted human serum and the bilirubin–HSA complex in water at neutral pH has been shown previously [6].

Previously suggested interpretations of the effects of pH and electrolytes on the CD spectra of bilirubin–HSA complexes included exciton coupling between electric transition dipole moments of the dipyrromethene chromophores of bound bilirubin [2]. In the case of a single bilirubin molecule, dissymmetric binding to a specific protein site was considered possible by rotation of the two bilirubin halves around the center methylene bridge. The variations in the magnitude and the sign of the CD bands observed under various conditions were then attributed to the formation of different conformers of bilirubin in each case. Thus, an inversion of the sign of the bands was correlated with the formation of sterically opposite positions of the transition dipole moments [1,2,9]. However, other contributions to the generation of the optical activity, such as those resulting from interactions of electric transition dipole moments of bound bilirubin with those of protein transitions, or inherent dissymmetry resulting from skewed chromophore conformations of the bound bile pigment, may also be important.

Attempts to resolve the positive CD curve shown in fig. 1 (3.5% 1-propanol) and the corresponding light-absorption spectrum (fig. 2) into a minimum number of Gaussian curves of similar parameters indicated a possible resolution into 5 Gaussians. For the CD spectrum, these constituted two pairs of exciton bands of opposite sign and, in addition, a larger positive band, centered near the observed band. The curves measured in water and in 6.25% 1-propanol could each be resolved reasonably well into three Gaussian curves. These curves were composed of an exciton couple and a positive band near 460 nm in each case. Further work is in progress for a more detailed analysis of these systems.

In view of the above-mentioned effects of 1-propanol and other alcohols and the general interpretation of the generation of optical activity in bilirubin–serum albumin complexes, it appears that these alcohols affect specific binding sites on the albumin. The alcohols may interact directly at the binding site, or they may affect the site indirectly by interacting with other parts of the protein. The short-chain alcohols may interfere effectively with hydrophobic interactions between bilirubin and the albumin. Such non-covalent interactions have been postulated previously for the bilirubin–BSA complex [10]. The role of electrostatic interactions has been demonstrated previously through the effects of pH and of electrolytes on the bilirubin–HSA complex [1,2,4,5]. The large influence of particular short-chain alcohols at relatively low concentrations suggests specific interactions, rather than general medium effects. Analogous phenomena have been reported relating to the action of alcohols on protein systems (e.g. [11]).

Acknowledgements

Thanks are due to Mrs. J. Silfen for her skilful technical assistance. This investigation was supported by grant No. 11 1772 from the Stiftung Volkswagenwerk.

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