A circular dichroism (CD) study of the consecutive binding of serum albumin to bilirubin. Possible implications for the bilirubin level

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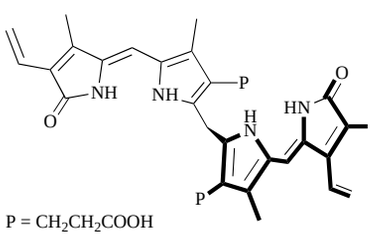
Binding studies on bovine serum albumin (BSA) with bilirubin (BR) have been performed under physiological conditions using circular dichroism (CD) and electronic absorption spectroscopy (UV–VIS). Analysis of data obtained from solutions at varying BSA/BR ratios (*R*), overall concentrations and pH suggests the coexistence of two similarly strong complexes with 1:1 stoichiometry ($K_{ass,M}$ *ca.* 1×10^7 dm^3 mol^{-1}). Further binding to BSA affords associates for which a 2:1 stoichiometry ($K_{ass,B}$ *ca.* 5×10^4 dm^3 mol^{-1}, pH = 7.4) is proposed. Results indicate that these aggregates comprise the main transport form of BR under physiological conditions. The role of the differently compounded BSA–BR complexes for controlling the concentration of unbound BR in the plasma is discussed. Preliminary experiments performed on human serum albumin (HSA) are in line with an analogous complexing behaviour.

Haem metabolism in mammals proceeds through the amphiphilic bilirubin (BR) which is solubilized by non-covalent, reversible binding to serum albumin (SA).[1–3] If continuous removal from plasma by reaction with glucuronic acid in the liver is hindered, the total concentration of BR increases thus provoking BR encephalopathy (BRE) which often occurs



BR

in neonates. BRE may cause irreversible mental disorder and even death. SA–BR complexes have therefore attracted the attention of many research groups in different disciplines for more than fifty years. It is now generally assumed that *in vivo* BR is present as a strong 1:1 associate with SA but the values reported for the most studied members human serum albumin (HSA) ($K_{ass,M} = 10^6$–10^8 dm^3 mol^{-1}) and bovine serum albumin (BSA) ($K_{ass,M} = 5 \times 10^6$–$2 \times 10^8$ dm^3 mol^{-1}) differ considerably.[1–9] In these complexes BR or a part of it is thought to be located in the hydrophobic crevice of subdomain IIA in which one propionate side chain forms a salt bridge with a basic amino acid.[3,10] A secondary weaker site for BR giving rise to a SA:BR = 1:2 complex at excess of BR over BSA has also been claimed,[1,3,4,7,11] but its existence could not be verified.†,[12] Furthermore it is believed that unbound BR causes BRE by passing the blood–brain barrier and penetrating the phospholipid membrane of neurones. However, BRE occurs even though SA is still in excess over BR[1,13,14] which leads to a dilemma considering the large binding constants reported for the 1:1 associate and the maximal increase in the concentration of unbound BR by a factor 60 on going to pathological conditions assuming $K_{ass,M} = 1 \times 10^7$ dm^3 mol^{-1}. Whether precipitation of BR induces BRE has become a matter of controversy.[13,15,16] Similarly, the increasing risk of BRE at lowered plasma pH has been explained differently.[1,9,13,15,16] Some authorities state a precipitation of the BR diacid (BH_2)[15,16] while more recent studies indicate an increase in the population of still soluble BH_2 on account of the BR monoanion (BH^-).[13] Thus, even though a considerable amount of excellent and thorough studies dealing with SA–BR complexes are available from the literature including CD monitored acidimetric studies,[1,6,17–19] conclusions to solve the vital questions are far from being consistent. In addition, the different conditions used in binding studies and the experimental difficulties encountered with the handling of BRs in general further complicate a combinatory evaluation of data. We have therefore initiated a systematic binding study on SA–BR complexes, in particular BSA–BR, under uniform and physiological conditions. We have chosen CD spectroscopy which is an established method in this area.[1,20] The induced CD (ICD) observed in the BR absorption region of SA–BR complexes is due to an overpopulation of chiral, kinetically labile BR conformers. Equipopulation of enantiomeric BR species becomes invalidated by the discriminating influence of the surrounding protein. This technique including scope and limitation have been critically examined recently.[20] Therefore, CD will mainly be used as a means to differentiate between associates and for the determination of binding constants.

Results and discussion

At pH = 7.4, solutions of BR and BSA in phosphate buffered saline (PBS) exhibit distinctly different ICD spectra depending on the ratio *R* of total molar concentrations [BSA]/[BR] (Fig. 1). For *R* values near unity the band ratio of the oppositely signed Cotton effects (CEs) centred at *ca.* λ 470 and 420 nm, respectively, amounts to *ca.* 3. On dilution the spectral phenotype ‡ does not substantially change and a straight line Scatchard graph (see Experimental) was obtained yielding $K_{ass,M}$ *ca.* 1×10^7 dm^3 mol^{-1} in agreement with several other values reported for the well known BSA–BR complex with 1:1 stoichiometry.[1,5] As the excess in BSA over BR increases up to $R = 10$ the CD band centred at λ *ca.* 420 nm becomes exceedingly weak while the diminution of the intensity of the long-wavelength band at λ *ca.* 470 nm is less pronounced (Fig. 1). Further addition of BSA results in minor changes. However, on dilution of this solution at constant *R* spectral changes become reversed approaching the ICD of the 1:1 associate (Fig. 2).

† The changes in CD spectra at excess of BR over BSA and HSA (R < 1), respectively, as reported in ref. 11 are rather due to precipitation of BR during titration.[12]

‡ For an interpretation of CD spectra in terms of conformation of the BR backbone see comments on p. 2122.

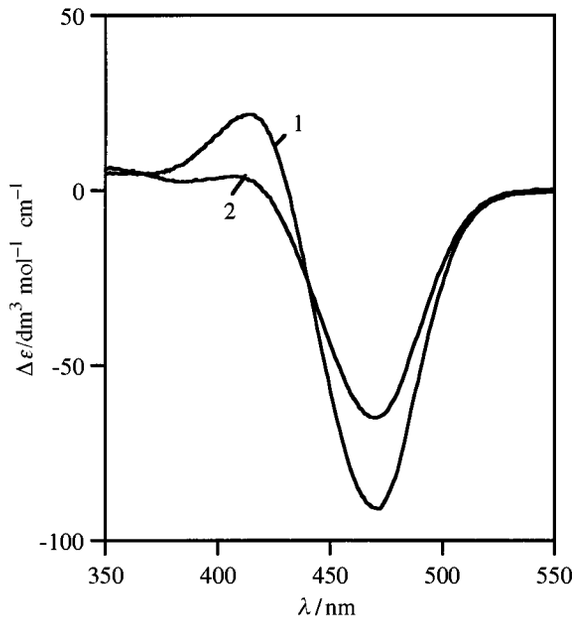


Fig. 1 CD monitored titration of BR with BSA in PBS buffer pH = 7.4. Solutions were 1.1×10^{-5} mol dm^{-3} in BR. To allow for a convenient comparison with Fig. 3, only spectra referring to $R = 1$ (1) and $R = 10$ (2) are shown. The isodichroic point appears at λ *ca.* 440 nm.

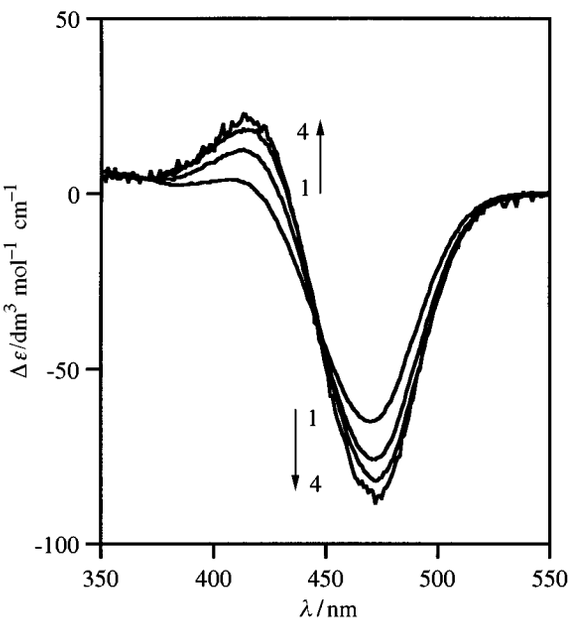


Fig. 2 ICD spectra for increasing dilution of BSA–BR solutions (PBS buffer, pH = 7.4) at $R = 10$. The concentrations of BR were 1: 1.10×10^{-5}, 2: 1.75×10^{-6}, 3: 7.02×10^{-7} and 4: 1.80×10^{-7} mol dm^{-3}

The course of titration along with the dilution experiments implies that the 1:1 associate initially formed can bind at least one additional BSA molecule. This subsequent binding is less strong and the associate formed decomposes more easily at lower overall concentrations into the 1:1 complex and BSA. An estimate of the apparent $K_{ass,B}$ of this complex was accomplished by Scatchard analysis from the CD monitored dilution at $R = 10$ at which dissociation of the 1:1 complex is negligible. By assuming 2:1 stoichiometry a straight line was obtained affording $K_{ass,B} = 5 \times 10^4$ dm^3 mol^{-1} which means that the beginning of the plateau region (R *ca.* 10) on titration of BR with BSA at pH = 7.4 [curve (1) Fig. 1] corresponds to *ca.* 80% of 2:1 associate.

In principle the stoichiometries of BSA–BR complexes occurring during the course of the titration of BR with BSA might also follow the order 1:2 ⟶ 1:1 instead of the 1:1 ⟶ 2:1 sequence as proposed here. Apart from other

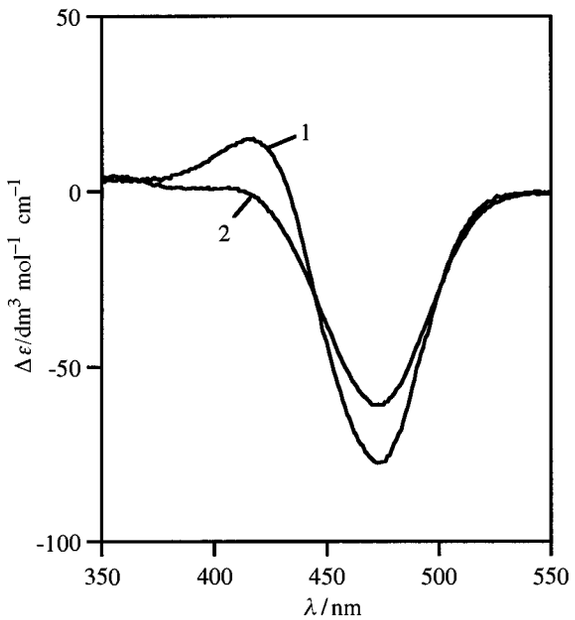


Fig. 3 ICD spectra of BSA–BR solutions at (1) pathophysiological concentrations [BSA (6×10^{-4} mol dm^{-3}), BR (4×10^{-4} mol dm^{-3}), $R = 1.5$] and (2) normal physiological conditions [BSA (6×10^{-4} mol dm^{-3}), BR (1×10^{-5} mol dm^{-3}), $R = 60$] in PBS buffer

indications against this alternative the crucial and distinguishing experiment comprises dilution studies (Fig. 2). The two ICD spectra (Fig. 3) both obtained at actual plasma concentrations (6×10^{-4} mol dm^{-3}) and a total BR content of *ca.* 2×10^{-5} and *ca.* 4×10^{-4} mol dm^{-3} for normal and pathological conditions, respectively, are close to those in Fig. 1 implying similarly different complexing modes at the respective two extreme R ratios. In agreement with computations (see below) taking into account the K_{ass} values determined, differences between spectra (2) in Figs. 1 and 3 are minute since under both conditions the higher aggregate predominates only rising from 80 to 95%. The significantly lower intensity of spectrum (1) in Fig. 3 compared with that in Fig. 1, in particular at λ *ca.* 470 nm, reflects more pronounced changes in the population of complexes. In fact, spectrum (1) in Fig. 3 refers to the tenfold overall concentration change and a slightly larger R value. Both factors promote consecutive binding and this accounts for an increase in the population of the 2:1 associate from less than 5 to *ca.* 50% (see below). It is also noteworthy that even at $R = 1.5$, and at pathophysiological total concentrations, the solubility limit of BR is not exceeded as revealed by the absence of light scattering of this solution (see Experimental).

We believe that neither of the ICD spectra displayed in Fig. 1 reflect the chiroptical properties of a single species. Rather, they each comprise an envelope of two spectra as can be judged from the distinct pH dependence of respective solutions. Accordingly, at R *ca.* 1 the ICD spectrum experiences a gradual change until pH *ca.* 8.1 at which the positive band at λ *ca.* 420 nm completely vanishes [Fig. 4(*a*)]. On lowering the pH to 7.0 the increase in intensity of the band at $\lambda = 420$ nm is more pronounced than that at $\lambda = 470$ nm and the band ratio decreases. Unfortunately, below pH = 7 increasing light scattering indicates precipitation of BR which prevents an estimation of the CD of this second species with 1:1 stoichiometry. On lowering the overall concentrations at any pH by dilution, the respective ratios of ICD bands are essentially maintained. Scatchard analysis in all cases yields a straight line and, most notably, differences in $K_{ass,M}$ values (*ca.* $1–2 \times 10^7$ dm^3 mol^{-1}) fall within the error of the method. Similarly, the ICD spectra (2) at $R = 10$ (Fig. 1) and $R = 60$ (Fig. 3) essentially representing 2:1 association must also be a population weighted average of at least two species. Increasing the pH under these conditions results in a decrease of the intensity of the single negative CE at λ *ca.* 460

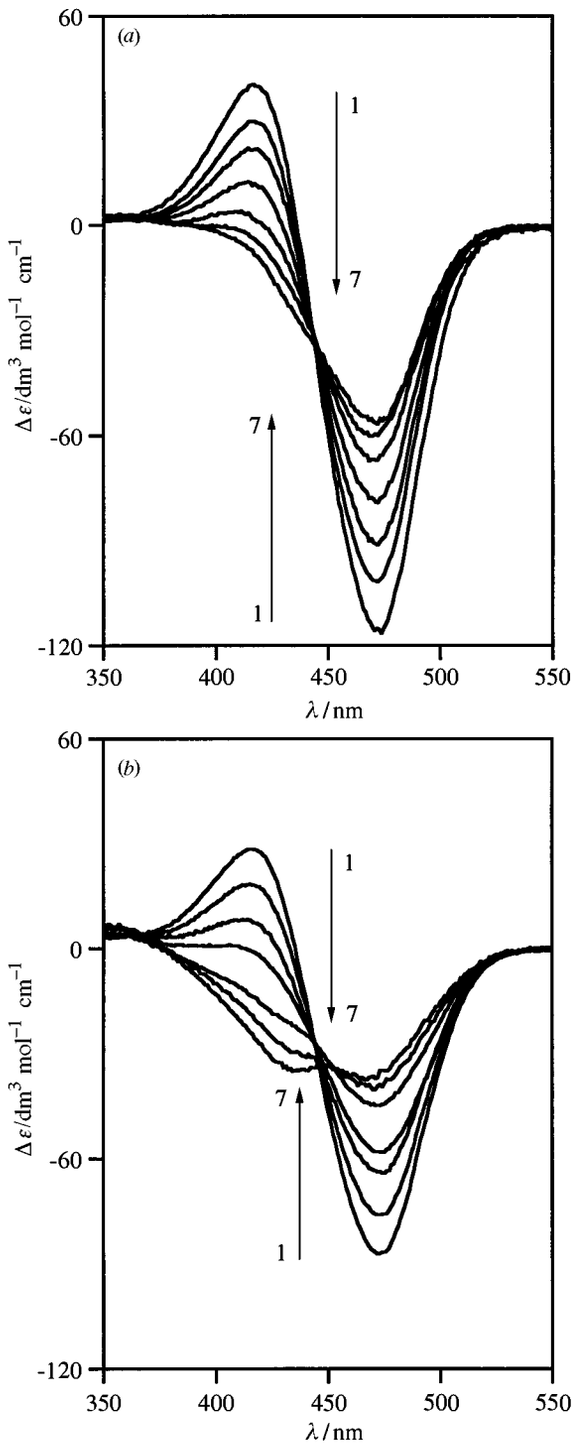


Fig. 4 pH-dependent ICD spectra of BSA–BR solutions at (*a*) $R = 1$ and (*b*) $R = 10$ in PBS buffer. Solutions were 1.10×10^{-5} mol dm^{-3} in BR; (*a*) pH values 1: 7.0, 2: 7.3, 3: 7.4, 4: 7.6, 5: 7.7, 6: 7.9, 7: 8.1; (*b*) 1: 7.1, 2: 7.2, 3: 7.3, 4: 7.5, 5: 7.8, 6: 8.0, 7: 8.1.

nm and the concomitant emergence of a second negative CE at λ *ca.* 435 nm [Fig. 4(*b*)]. Thus the final spectrum (pH *ca.* 8.1) is quite different from that observed at $R = 1$. On the other hand, below pH = 7.4 the single negative CE at λ *ca.* 470 nm gradually transforms into an exciton couplet showing an additional, oppositely signed band at λ *ca.* 420 nm. However, the intensity of bands, in particular those at λ *ca.* 470 nm, is rather weaker than that observed at $R = 1$. On dilution of solutions at constant pH the shape of spectra displayed in Fig. 4(*b*) turns into those as obtained at $R = 1$ and the same pH as in Fig. 4(*a*).

Unfortunately, the question about the pH dependence of $K_{ass,B}$ for additional binding of BSA to the primary formed 1:1 complexes cannot yet be answered conclusively because of the considerable scattering of values, in particular above pH = 7.4, yielding $K_{ass,B}$ values in the range from 1×10^4 to 1×10^5 dm^3 mol^{-1}.

It must be emphasized that even if some of the spectra in Fig. 4(*a*) ($R = 1$) and Fig. 4(*b*) ($R = 10$) recorded at different pH values resemble each other (*e.g.* at $R = 10$, pH = 7.5 and $R = 1$, pH = 7.9) they reflect different distributions of associates as follows from their distinct behaviour on dilution of the corresponding solutions.

Qualitatively the same spectroscopic phenomena as described above for the BSA preparation used in the foregoing experiments (97–99% BSA) were obtained for fatty acid-free BSA, *S*-cysteinyl-Cys[34]-BSA and for the native dimer.§ They differ only in that comparable changes of spectra with varying R occur at slightly (by *ca.* 0.1–0.2 units) higher pH.

This conformity rules out the spectral changes described in the foregoing being due to an inhomogeneity stemming either from partial blocking of the free sulfhydryl group of Cys[34] or from the presence of oligomers in the BSA preparation used. The remarkable pH dependent population of BSA–BR associates raises the question of its origin. If BR binds to BSA only as a dianion B^{2-} a pH dependent equilibrium between two complexes differing in the charge of their BSA components [eqn. (1)] must exist. Since the 'neutral (N) to base (B)' transition[21] of SAs occurs within the pH range investigated it is tempting to correlate BSA^{m-} with the N-form and $BSA^{(m+n)-}$ with the B-form, n being the Hill coefficient which has been estimated to be close to unity. Since B^{2-} is composed of two pyrrinone halves each carrying a propionate side chain, both 1:1 associates may bind an additional BSA molecule even if less strongly. Thus in analogy to affinity chromatography BR serves as a spacer between two macromolecules resulting in the 2:1 stoichiometry. This model implies two pairs of BSA–BR complexes with 1:1 and 2:1 stoichiometry, respectively.

$$BSA^{m-} \rightleftharpoons BSA^{(m+n)-} + nH^+ \qquad (1)$$

Alternatively, the pH-dependent CD spectra might arise from the differently charged BR species BH^- and B^{2-}, respectively [eqn. (2)]. At the present time we are unable to make a clear decision between these two approaches as the number of protons involved in the individual equilibria is unknown. Besides, a combination of the two possibilities cannot be excluded. Even if an unequivocal proof for the distribution of charge within the complexes is still lacking, it is evident that any kind of additional binding of BSA to a 1:1 complex already formed would further lower the concentration in unbound BR.¶ This effect increases with increasing excess in BSA and increasing association tendency of consecutive complexation. Fig. 5 shows a comparison of the calculated dependence of the concentration of unbound BR on R at pH = 7.4 and at physiological BSA concentrations assuming 1:1 association only, and the consecutive formation of a 2:1 associate, respectively. At $R = 1.5$ concentrations are almost identical for the two complexing modes but as R increases differences become pronounced. At $R = 60$ the concentration of unbound BR is further reduced by a factor 30 due to consecutive binding. This rather lower concentration would in principle correspond to a 1:1 binding constant of $K_{ass,M}$ *ca.* 3×10^8 dm^3 mol^{-1}. Fig. 6 shows a plot of the relative population of BSA–BR complexes

$$BH_2 \rightleftharpoons BH^- + H^+ \rightleftharpoons B^{2-} + 2\,H^+ \qquad (2)$$

§ The CD spectra in the amide absorption region of all four BSA preparations are very similar and prove to be essentially independent of pH within the range 6–9 (see Experimental).

¶ Only at $R < 1.5$ does the competition between the 1:1 and 2:1 associate for BSA result in an increase in the concentration of unbound BR if compared with exclusive 1:1 association.

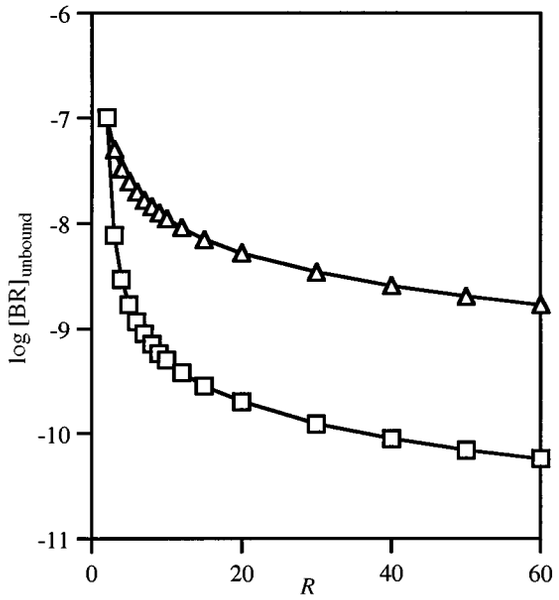


Fig. 5 Computed concentrations of unbound BR, $[BR]_{unbound}$ (logarithmic scale) *versus* R (R = 1.5–60) at pH = 7.4 for BSA : BR = 1 : 1 association only (apparent $K_{ass,M} = 1 \times 10^7$ dm^3 mol^{-1}) (△) and for the additional involvement of a 2 : 1 complex (apparent $K_{ass,M} = 5 \times 10^3$ dm^3 mol^{-1}) (□), respectively, at physiological BSA concentrations

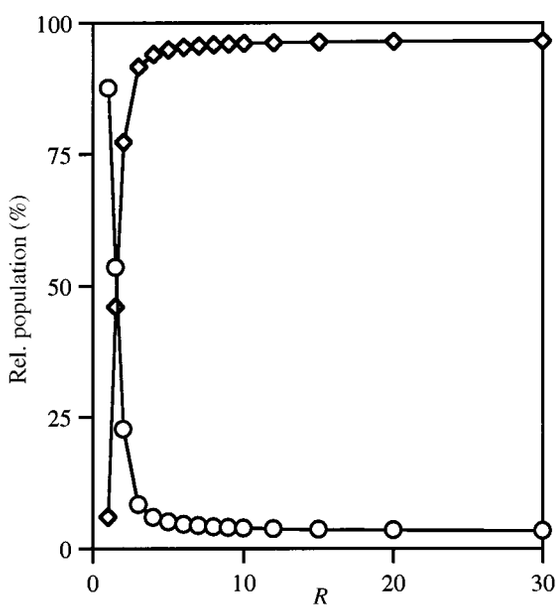


Fig. 6 Computed relative populations (%) of BSA–BR associates *versus* R(R = 1–30) for consecutive binding of BSA to BR at physiological BSA concentrations; 2 : 1 complex (◇), 1 : 1 complex (○); K_{ass} values and conditions are the same as in Fig. 5

with 1 : 1 and 2 : 1 stoichiometry, respectively, as a function of R. The proportion of unbound BR (not shown) is as low as 1.5% at R = 1.5 and becomes negligibly small at larger R ratios. The relative amount of complexes changes until R *ca.* 10 but becomes essentially invariable above this limit.

It seems appropriate at this point to comment on the ICD spectra (Figs. 1–4) in more detail. Considering the CD monitored acidimetries, displayed in Fig. 4, it becomes apparent that those complexes which are favoured at lower pH must exhibit an exciton couplet differing only in the intensity of bands. On the other hand, one and two negative CEs, respectively, are characteristic of those complexes which predominate at pH *ca.* 8.1. Therefore one might speculate about the kind of conformational transition in BR underlying these spectral changes. However, apart from the fact that most of the spectra displayed in Figs. 1–4 comprise superpositions of different and differently weighted components, the diagnostic value of ICD in this area *per se* is poor, since neither the extent of chiral discrimination between enantiomeric *M* and *P* forms of bilirubin BRs by BSA in the individual complexes, nor the spectral differences between diastereoisomers are known.[20] Besides, an exciton couplet may reflect quite different angles between the mean planes of the two dipyrrinone moieties.[22] Similar arguments hold true for a single (negative) CD band. Its occurrence implies only that the two chromophoric constituents of BR are electronically independent but this may occur in a closed helical as well as in an extended conformation. The (negative) double band due to the 2 : 1 associate at pH = 8.1 may arise from different twisting of the two electronically independent, isomeric dipyrrinone chromophors or even may indicate an inhomogeneity of this complex under these conditions. However, it is not due to the unsymmetrical substitution pattern of BR since the symmetric XIIIα-isomer shows the same spectral behaviour. UV–VIS spectroscopy comprises a more adequate method in the conformational analysis of BRs.[22,23] Thus the predominance of the long wavelength band at λ *ca.* 470 nm over that at λ *ca.* 420 nm (shoulder) which is characteristic for all UV–VIS spectra of BSA–BR solutions under any conditions suggest that BR in none of the associates adopts a closed helical conformation. Rather, in all complexes BR belongs to a closely related rotameric family of extended conformers.

The great similarity of BSA to HSA, sharing high sequence homology and tertiary structure including the three domains and six subdomains along with their closely related functions,[3] suggests a similar complexing behaviour. The toplogical resemblance of their peptide backbones is also reflected in almost superimposable CD spectra (see Experimental). In fact, preliminary experiments performed on HSA–BR complexes are in favour of related binding modes as deduced for BSA–BR. Thus, straight line Scatchard graphs by the dilution method were obtained only at R *ca.* 1, while at R > 2 plots are invariably curved indicating the coexistence of a species in which HSA is in excess. In his careful work Beaven[4] has also found two HSA–BR complexes in the course of titration of BR and HSA but assigned to them the different stoichiometries. However, the 1 : 1 complex predominates at R values near unity rather than at R > 3 as can easily be judged from dilution experiments. Therefore the 1 : 2 and 1 : 1 stoichiometries proposed in ref. 4 should be replaced by 1 : 1 and 2 : 1. The binding constant $K_{ass,M}$ *ca.* 1×10^7 dm^3 mol^{-1} evaluated at pH = 7.4 is indistinguishable from that obtained for BSA–BR 1 : 1 association. Unfortunately, two main factors make a more thorough investigation of HSA–BR complexes by CD spectroscopy more difficult. Firstly, the ICD response under the conditions chosen is two to five times lower than that observed with BSA–BR complexes. Secondly, provided that in fact a similar distribution of species exists as with BSA–BR, the spectral differences of the corresponding HSA–BR associates must be less pronounced. Thus, only a decrease in the overall intensities of the CD couplets is observed with increasing R from 1 to 60 or increasing pH from 7 to 8.‖

Conclusions

We have shown that binding of BR to SA comprises a complex process in which a variety of associates may coexist. The considerable sensitivity of their population towards a variation in R ratios, pH and overall concentrations even within the narrow physiologically relevant range investigated in this study may provide a rationale for the inconsistencies in K_{ass} values, spectral properties and binding capacities reported for SA–BR complexes. The two species with 1 : 1 stoichiometry exhibit similar binding constants, which in turn comprises the basis for the finding that the $K_{ass,M}$ value of the SA–BR complex which has

‖ Fatty acid-free HSA was used.

been considered homogeneous until now is independent[9] of pH. Our results further suggest the existence of associates in which SA is in at least twofold excess over BR (2:1 complex). Since the normal physiological excess in total SA (*ca.* 6×10^{-4} mol dm^{-3} corresponding to *ca.* 3.5 g/100 cm^3) over total BR (*ca.* $1–2 \times 10^{-5}$ mol dm^{-3} corresponding to 0.5–1.0 mg/100 cm^3) varies between $R = 30$ and 60 our results imply that under these conditions BR is mainly associated with two molecules of SA rather than with one. This might be of outstanding importance since the concentration of free BR, which would amount to 1.7×10^{-9} mol dm^{-3} if only an 1:1 associate occurred becomes as low as 5.7×10^{-11} mol dm^{-3} owing to the additional involvement of 2:1 associates (Fig. 5). Hence, subsequent binding of BSA to the 1:1 associates provides for a further decrease in the concentration of unbound BR by a factor of 30. On the other hand, under conditions at which BRE occurs ($2–4 \times 10^{-4}$ mol dm^{-3} total BR corresponding to 10–20 mg/100 cm^3) R becomes as low as 1.5–3 and a redistribution of species in favour of the two 1:1 associates and unbound BR takes place. In this case, however, no substantial reduction of the concentration in unbound BR, amounting to *ca.* 1×10^{-7} mol dm^{-3}, takes place. Accordingly, the concentration of unbound BR increases by a factor of 1800 rather than by a factor of 60 by changing from normal to pathophysiological conditions. Significant penetration of BR into the lipophilic tissues becomes more plausible in view of these striking differences. But even at pathophysiological total concentrations of BR the unbound portion does not exceed the solubility limit. This is in favour of the statement[13] that dissolved BR rather than precipitated BR induces BRE.[13]

Experimental

General

BR (Serva) and its XIIIα-isomer, as obtained by acidic scrambling, were chromatographed on silica gel as described.[24] Serum albumins [BSA (97–99%, A 4378), BSA (fatty acid-free, A 7511 and A 0281), *S*-cysteinyl-Cys[34]-BSA (A 0161), BSA-dimer (A 9039)[26] and HSA (fatty acid-free, A 3782)] were all from Sigma and were used as purchased. The molecular masses assumed were $M = 66\,500$ and $M = 67\,000$ for BSA and HSA, respectively. Measurements were performed in phosphate buffered saline (PBS) containing 4 mmol dm^{-3} KH_2PO_4 (p.A. Merck), 16 mmol dm^{-3} Na_2HPO_4 (p.A. Merck) and 121 mmol dm^{-3} NaCl (p.A. Merck). The water used throughout this study was distilled twice. The pH was appropriately adjusted (±0.05 units) with 1 mol dm^{-3} NaOH or 1 mol dm^{-3} HCl using a Metrohm 691 pH meter. The glass electrode was calibrated by using two buffer solutions (Metrohm). pH values given in the text are rounded to the first decimal place. Prior to use buffers were sonicated for 15 min and then flushed with argon. Solutions of BR and SAs were protected from light and air. CD spectra were run with a CD6 circular dichrograph (I.S.A. Jobin-Yvon) in thermostatted quartz cuvettes at variable path length (0.02–10 cm) at 20 ± 1 °C. Each spectrum is the average of 2–5 consecutive scans. Baseline corrections between $\lambda = 350–600$ nm were provided by PBS solutions at the appropriate BSA concentrations and pH values. The SAs were CD inactive within this wavelength region. UV–VIS spectra were recorded with a Perkin-Elmer Lambda 7 instrument. All measurements described below were run at least in triplicate and refer to freshly prepared solutions which were used after 15–30 min. If not stated otherwise spectra were then independent of time for 2 h. Several titrations which could not be finished within this time were obtained in a discontinuous manner on repeatedly freshly prepared solutions. The experiments described below refer to BSA [97–99%, A 4378, Sigma, λ_{max}/nm (PBS) 222 ($\Delta\varepsilon = -3740$ dm^3 mol^{-1} cm^{-1}) and 208 (−3900)] and natural BR as mainly used in this study. The content of free sulfhydryl groups of SAs was occasionally determined using 4,4′-dithiodipyridine[27] and afforded 0.60 and 0.18 SH per molecule for BSA (A 4378) and HSA (A 3782), respectively. Investigations on fatty acid-free BSA [λ_{max}/nm (PBS) 222 ($\Delta\varepsilon = -3600$ dm^3 mol^{-1} cm^{-1}) and 209 (−3800)] and HSA [λ_{max}/nm (PBS) 222 ($\Delta\varepsilon = -3640$ dm^3 mol^{-1} cm^{-1}) and 209 (−3880)], *S*-cysteinyl-Cys[34]-BSA [λ_{max}/nm (PBS) 222 ($\Delta\varepsilon = -3490$ dm^3 mol^{-1} cm^{-1}) and 209 (−3700)], the native, dimeric BSA [λ_{max}/nm (PBS) 222 ($\Delta\varepsilon = -3530$ dm^3 mol^{-1} cm^{-1}) and 209 (−3710), values refer to the monomeric unit] and BR XIIIα, respectively, were performed analogously and afforded similar results. The CD spectra of proteins given in the above refer to pH = 7.4 but changes of magnitudes in the pH range 6–9 do not exceed 3%. For changes in the absorption region of the aromatic amino acid side chains see refs. 1 and 21.

In the following, binding studies are only described for the BSA monomer and natural BR-IXα. Investigations performed on the other SAs and BR-XIIIα were appropriately adapted.

CD monitored titrations

Solutions A–C were prepared as follows. Solution A: BSA (100 mg, 1.5×10^{-6} mol) was dissolved in twice distilled water and brought to a volume of 0.5 ml, which refers to a 3.01×10^{-3} mol dm^{-3} solution. Solutions B and C: BR (0.130 mg, 2.2×10^{-7} mol) was rapidly dissolved in 0.025 mol dm^{-3} KOH (0.1 cm^3) followed by addition of PBS buffer (0.4 cm^3). This solution is unstable and soon becomes cloudy most likely due to precipitation of BR dipropionate oligomers. 0.25 cm^3 of the still clear solution were then added to PBS buffer (3 cm^3) containing BSA [7.4 mg (1.1×10^{-7} mol) and 74.0 mg (1.1×10^{-6} mol) for solution B ($R = 1$) and C ($R = 10$), respectively] and filled up to a total volume of 10 cm^3. Solution B contains 1.1×10^{-5} mol dm^{-3} BR and BSA, respectively ($R = 1$). Solution C contains the tenfold amount (1.1×10^{-4} mol dm^{-3}, $R = 10$) of BSA. pH adjustment was performed by addition of 1 mol dm^{-3} NaOH or 1 mol dm^{-3} HCl.

Variation of *R* at constant pH. The pH value of solution B was appropriately adjusted. 2.5 cm^3 of this solution were then transferred into a 1 cm quartz cuvette equipped with a magnetic stirrer. Titration was performed by injecting the appropriate volume of solution A (9.2 mm^3 correspond to 1 equiv. BSA), step by step, directly into the cuvette followed by a CD and UV–VIS study. Values were corrected for volume increase. At large R values the pH had to be readjusted owing to the slight acidity of solution A.

For measurements at pathophysiological ($R = 1.5$) and normal physiological ($R = 60$) overall concentrations, solution D was prepared by dissolving BR (0.461 mg, 7.88×10^{-7} mol) in 0.025 mol dm^{-3} KOH (0.2 cm^3) followed by addition of PBS buffer (0.8 cm^3). 0.5 cm^3 (3.94×10^{-7} mol BR) of solution D was then added to PBS buffer (*ca.* 0.4 cm^3) containing 200 mm^3 (6.02×10^{-7} mol BSA) of solution A and filled up to 1 cm^3 with PBS buffer. This mixture contains 3.94×10^{-4} mol dm^{-3} BR and 6.02×10^{-4} mol dm^{-3} BSA, respectively, and refers to $R = 1.5$. The solution was adjusted to pH = 7.4 then turbidimetrically checked for precipitated BR and subjected to a CD measurement using a cuvette of 0.02 cm path length.

For normal physiological overall concentrations ($R = 60$) 63 mm^3 (4.96×10^{-8} mol BR) of solution D were injected into a solution of BSA (200 mg, 3.01×10^{-6} mol) in PBS buffer (3 cm^3) and brought to a volume of 5 cm^3. This solution contains 6.02×10^{-4} mol dm^{-3} BSA and 1.00×10^{-5} mol dm^{-3} BR corresponding to $R = 60$. Turbidimetry revealed the absence of undissolved components. After adjustment to pH = 7.4 the CD spectrum was recorded using a cuvette of 1 cm path length.

Variation of pH at constant *R*. Solutions B and C, respectively, each *ca.* 3 cm^3, were titrated in an 1 cm quartz cuvette by injection of 1 mol dm^{-3} HCl and 1 mol dm^{-3} KOH (0.2–2 mm^3, for 0.1–0.2 pH intervals). The pH value was measured directly in the cuvette with a glass electrode followed by recording the

CD and UV–VIS spectra. At the end of titrations the reversibility of spectra was checked by readjustment to the initial pH 7.4. Ageing of solutions in particular at $R = 1$ (solution B) is accelerated above pH 7.8. For this reason the complete pH range was obtained in a discontinuous manner by using up to three freshly prepared solutions. The ageing at $R = 1$ is distinguished by similar spectral changes as observed at $R = 10$ which are, however, irreversible.

Variation of overall concentration at constant *R* and pH, determination of apparent binding constants $K_{ass,M}$ and $K_{ass,B}$. Solutions B and C, respectively, were adjusted to the desired pH and then diluted with PBS buffer of the same acidity. CD and UV–VIS measurements were performed in quartz cuvettes of variable path length (0.5–10 cm). The Scatchard method[20,28] was used for the evaluation of apparent K_{ass} values. Straight lines were obtained by applying the appropriate stoichiometry. Additionally, a modified procedure[29] based on linear extrapolation of CD parameters to infinite concentration was used as described below in more detail. For the determination of $K_{ass,M}$ of the BSA–BR 1:1 complexes at given pH, differently diluted solutions B in the range 1.1×10^{-5}–6×10^{-8} mol dm^{-3} were subjected to CD measurements. From the plot $1/\Delta\varepsilon_\lambda$ *versus* $1/[BR]_{total}$ at $\lambda = 470$ and/or $\lambda = 410$ nm the absolute $\Delta\varepsilon_{\lambda,M}$ values were obtained from the *y* intercept $1/\Delta\varepsilon_{\lambda,M}$ while the *x* intercept $-RK_{ass,M}$ afforded $K_{ass,M}$. Analogously, the $K_{ass,B}$ values for the 2:1 complexation at given pH were obtained through successive dilution of solution C followed by a CD measurement. The plot $1/(\Delta\varepsilon_\lambda - \Delta\varepsilon_{\lambda,M})$ *versus* $1/[BR]_{total}$ afforded the absolute $\Delta\varepsilon_{\lambda,B}$ value from the *y* intercept $1/(\Delta\varepsilon_{\lambda,B} - \Delta\varepsilon_{\lambda,M})$ while $K_{ass,B}$ was taken from the *x* intercept $-(R - 1)K_{ass,B}$.

Computations

The dependence of the concentrations of unbound BR and the complexes with 1:1 and 2:1 stoichiometries, respectively, at pH = 7.4 and physiological BSA concentrations (6×10^4 mol dm^{-3}) on *R* ratios was determined by an iterative approach using a modified procedure as described.[30] Accordingly, concentrations of components involved in $K_{ass,M}$ for 1:1 association [eqn. (3)] were fitted to those involved in 2:1 association [eqn. (4)].

$$K_{ass,M} = \frac{[BSA{\cdot}BR]}{[BR][BSA]} \quad (3)$$

$$k_{ass,B} = \frac{[BSA_2{\cdot}BR]}{[BSA{\cdot}BR][BSA]} \quad (4)$$

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