Maximum binding capacity of serum albumin for bilirubin is one, as revealed by circular dichroism

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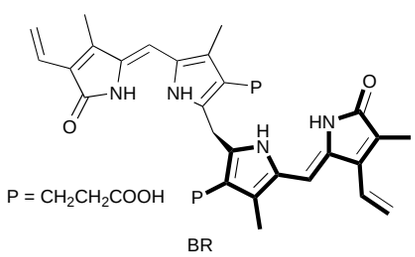
A reinvestigation of the binding capacity of bovine serum albumin (BSA) and human serum albumin (HSA), respectively, for bilirubin (BR) at pH 7.4 is presented using circular dichroism (CD), UV–VIS spectroscopy and light scattering measurements. Evidence is provided that mixtures of either SAs with BR do not form true solutions in aqueous buffer if the excess of BR over SA exceeds *ca.* 1.4 equivalents. The study throws doubt on multiple BR binding onto SA at pathophysiological conditions and on the significance of this process for lowering the concentration in unbound BR.

Introduction

Serum albumin (SA) serves as a vehicle for the heme metabolite bilirubin (BR) which is transported to the liver for



BR

detoxification.[1–3] Owing to tight binding of the BR mono- and/or di-propionate to SA †,[1–5] at the primary site in a 1 : 1 manner (K_{ass} *ca.* 1×10^7 dm^3 mol^{-1})[5–7] the concentration of free, cytotoxic BR is kept low. Recently we have shown that at normal physiological conditions at which SA is in about 30–60-fold excess over BR subsequent binding of the protein onto the 1 : 1 associate furnishes a higher aggregate for which a 2 : 1 stoichiometry (K_{ass} *ca.* 5×10^4 dm^3 mol^{-1}) seems likely.[5] The additional equilibrium between the 1 : 1 and 2 : 1 complexes causes a further decrease in the concentration of free BR by a factor of 30 while at pathophysiological BR overall concentrations the 2 : 1 complex has no influence. This distinctly different population of SA–BR 2 : 1 complexes on going from normal to pathophysiological conditions brings about an exceptionally large difference in the concentration of unbound BR by a factor of *ca.* 1800.[5] The development of kernicterus in new born infants becomes thus more plausible in view of such striking changes. However, the considerations outlined are valid if and only if additional binding of BR onto the 1 : 1 complex can be neglected. Clearly, although it is generally accepted that multiple BR binding onto SA is irrelevant at normal SA : BR ratios, SA–BR complexes of 1 : 2 or even higher stoichiometry would also cause a lowering in the concentration of unbound BR but, differently from the 2 : 1 complex, at pathophysiological BR levels.[1] While the existence of a single high affinity site of SAs for BR is well established the agreement about the existence of additional sites distant from the primary one is poor.[1] Up to 20 secondary sites have been proposed using partition, spectroscopic ‡ and kinetic techniques, and even unspecific binding has been reported.[1,6,8–12] We suspected that the reason for the dissensions and inconsistencies inherent in investigations on multiple BR binding might arise from the fact that studies on this subject have been carried out at an excess of BR over SA to promote additional BR binding. This, however, can only be achieved by supersaturation considering the poor solubility of BR in the absence of stoichiometric amounts of SA in aqueous media at physiological pH.[1,4,8] On the other hand there are strong indications that BR in buffer solutions alone or in the presence of insufficient amounts of SA is no longer monomeric.[1,9] This prompted us to reinvestigate the binding capacity of the two main SA members bovine serum albumin (BSA) and human serum albumin (HSA) for BR in more detail. For both of them secondary sites have also been deduced from circular dichroism (CD) measurements[11,12] ‡ and this technique will be used in the following study. In this context it seemed advisable to make additional allowance for results obtained from UV–VIS and light-scattering measurements.

As has been outlined elsewhere[9,13,14] in more detail, the occurrence of a CD signal in the BR absorption region of SA–BR mixtures relies upon the discriminating influence of the homochiral, kinetically stable SA upon the kinetically labile racemic mixture BR by provoking the formation of differently populated diastereoisomeric complexes. Hence, the CD signal observed is induced (ICD) by the host SA and differs in origin from natural optical activity.[15]

Results and discussion

On addition of one equiv. of an alkaline BR solution to BSA dissolved in phosphate buffered saline (PBS) at pH 7.4 the CD intensity at λ *ca.* 470 nm (negative) is three times larger than that at λ *ca.* 410 nm (positive) indicating the predominance[5] of 1 : 1 association [Fig. 1(*a*), curve (1)]. No substantial changes are observed unless the molar excess in the ratio (r) of BR over BSA exceeds *ca.* 1.5. If addition of BR is continued beyond this limit the two Cotton effects (CEs) experience differently strong diminutions and at r *ca.* 4 the CE centered around λ 410 nm splits into two components. Further spectral changes occur beyond $r = 5$ including inversion of signs of the main CEs at $r = 8$ (not shown). If pure phosphate buffer (PB) is used instead of PBS buffer spectral changes are more pronounced but qualitatively the same. Analogous experiments were also carried out with HSA. During this titration the two CD bands due to the HSA–BR 1 : 1 complex centered at λ *ca.* 415 nm (negative) and λ *ca.* 470 nm (positive), opposite in sign if compared with the CEs

† Throughout this paper the notation BR is used without specification of the actual ionic state which may by different in the bound and unbound molecules.

‡ The SA–BR 1 : 2 and 1 : 1 complexes claimed in ref. 11 possess instead 1 : 1 and 2 : 1 stoichiometries, respectively.[5]

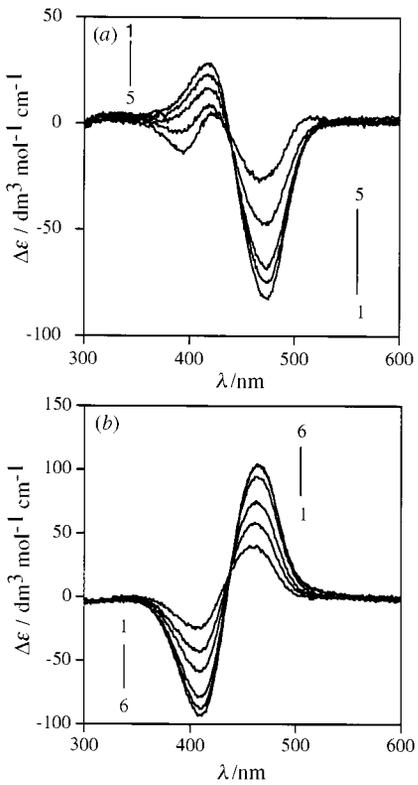


Fig. 1 Titration of (*a*) BSA and (*b*) HSA with BR in PBS buffer at pH 7.4 as monitored by CD spectroscopy at 293 K. Solutions were 4×10^{-6} mol dm^{-3} in BSA and HSA, respectively; $\Delta\varepsilon$ values of all CD spectra in the BR absorption region refer to this concentration (*a*) *r* values (1): 1; (2): 2; (3): 3; (4): 4; (5): 5; (*b*) *r* values (1): 1; (2): 1.5; (3): 2; (4): 3; (5): 4; (6): 5.

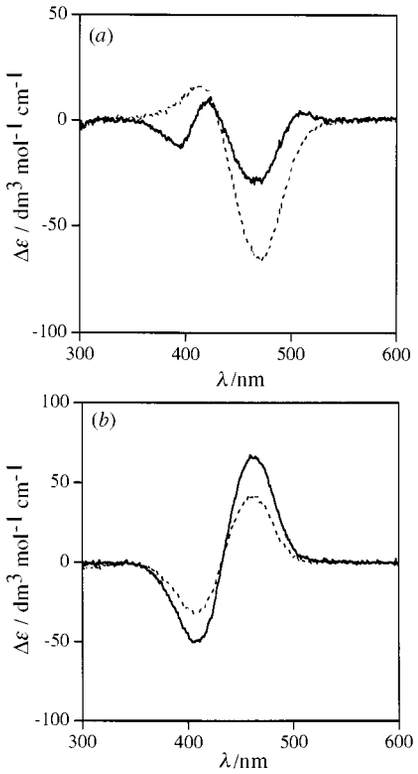


Fig. 2 CD spectra of (*a*) a BSA–BR mixture at $r = 5$ and (*b*) a HSA–BR mixture at $r = 3$ before (——) and after (– – –) centrifugation; other conditions were the same as in Fig. 1

of the BSA–BR 1 : 1 complex,§ proportionally increase in magnitude up to *r ca.* 4 but then become almost invariant [Fig. 1(*b*)]. Unlike the corresponding titration of BSA with BR the spectral phenotype does not depend on the amount of BR added. No difference in the course of titration between PB and PBS buffer is observed. Similar variations in CD spectra on addition of BR to BSA and HSA, respectively, have been taken as proof of the existence of additional binding sites of SAs for BR [12] which are believed to become active at *in vivo* conditions.

However, even on immediate readjusting of solutions containing more than *ca.* 1.5 equiv. BR to $r = 1$ by addition of the corresponding SA the absolute magnitudes of CD spectra expected for the respective 1 : 1 complexes on the basis of overall concentrations are no longer obtained. For example, although the composite CD spectrum of the BSA–BR solution at $r = 5$ changes into that of the 1 : 1 complex, $\Delta\varepsilon$ values are distinctly lower. This discrepancy increases as the excess in BR of solutions subjected to retitration increases and amounts to *ca.* 10% starting from $r = 1.8$ and to *ca.* 50% starting from $r = 5$. Moreover, both CD and UV–VIS spectra of solutions containing more than 1.5 equiv. BR change with time while concomitantly the fraction of BR reconvertable into the 1 : 1 complex further decreases. This time dependence consists of a slow reversion of all spectral changes originally brought about by addition of BR in excess over one equiv. SA. Thus the CD intensities around λ 410 nm and 470 nm exhibited by BSA–BR and HSA–BR mixtures at $r > 1.5$ increase and decrease, respectively, while in both cases a loss in absorbtivity at λ *ca.* 460 nm occurs. Unfortunately, at pH 7.4 complete reversion of spectral features to those observed at $r = 1$ was not reached within 24 h.¶ Since BR may undergo interfering side reactions over 24 h or longer periods, ageing was accelerated by moderate centrifugation for 2 h followed by gentle shaking. By this procedure the visible absorption band due to BR at λ *ca.* 460 nm of solutions at $r = 1$ is not affected but at larger *r* values a striking absorbance decay takes place (Table 1). In particular for BSA–BR solutions containing a three- and five-fold excess of BR the ageing process seems already to be complete since only about 1 equiv. is left in solution. Clearly, if the concentrations of BR are treated as equal with that of BSA which is equivalent to multiplying the apparent extinction coefficients with the corresponding *r* value, the ε values (63 000 and 70 000 dm^3 mol^{-1} cm^{-1}, respectively) thus obtained are close to that of the 1 : 1 complex (59 800 dm^3 mol^{-1} cm^{-1}). Analogous variations are observed by CD spectroscopy but as above solutions at $r = 1$ are not affected. Thus the multiple CD characteristic of freshly prepared BR–BSA solutions at $r = 5$ [Fig.1(*a*)] changes into an exciton couplet associated with the phenotype of the BR–BSA 1 : 1 complex [Fig. 2(*a*)], while the CD bands of BR–HSA solutions which increase in intensity with increasing *r* [Fig. 1(*b*)] become less intense on ageing or centrifugation [Fig. 2(*b*)].

The experiments described in the previous paragraphs suggest that after addition of more than *ca.* 1.5 equiv. BR to SA solutions the residual BR which adopts a metastable aggregate state [9] currently precipitates. Therefore changes in CD spectra of SA–BR mixtures by increasing the ratio *r* beyond 1 seem to arise from temporary binding of dimeric and/or oligomeric BR onto the SA–BR 1 : 1 complex. On ageing, the size of aggregates increases and finally BR precipitates causing the CD active SA bound oligomers to decompose into the differently CD active 1 : 1 associate and inactive BR. This interpretation accounts

§ These differences in CD spectra are in fact of low diagnostic value [13] and simply mean that the highly flexible BR differently fits its conformation to the respective topology of the hydrophobic crevice located in subdomain IIA of the hosts BSA and HSA. Even if conformations were enantiomeric to each other the binding constants of 1 : 1 associates are not affected as they are of the same order.[5,13]

¶ The stability of the BR sol along with the aggregates capable of binding to the 1 : 1 complex decreases with decreasing pH so that at pH 7.1 ageing becomes considerably enhanced. On the other hand, on raising the concentration of SA from 4×10^{-6} to 7×10^{-5} mol dm^{-3} at constant *r* ageing is significantly retarded.

Table 1 UV–VIS spectra (λ_{max}/nm) of various SA–BR mixtures in PBS buffer at pH 7.4 and 293 K before and after centrifugation. ε Values refer to the total BR content. Solutions were 4×10^{-6} mol dm^{-3} in BSA and HSA, respectively.

BSA			HSA		
	ε_{max}/dm^3 mol^{-1} cm^{-1}			ε_{max}/dm^3 mol^{-1} cm^{-1}	
r	Before	After	r	Before	After
1	59 800 (462)	58 000 (461)	1	47 500 (461)	48 000 (458)
2	53 300 (454)	40 000 (458)	1.6	44 000 (457)	33 600 (458)
3	48 900 (449)	21 000 (459)	3	44 600 (456)	22 500 (457)
5	47 400 (442)	14 000 (460)	4	41 400 (453)	—

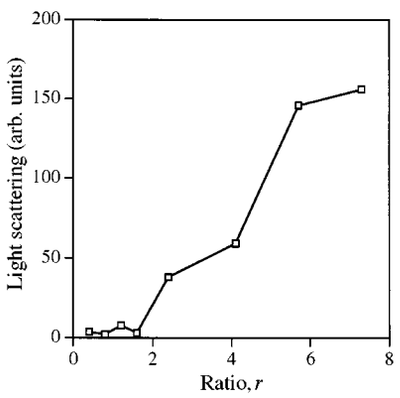


Fig. 3 Light scattering (arbitrary units) at λ 560 nm during titration of BSA ($c = 4 \times 10^{-6}$ mol dm^{-3}) with BR; other conditions were the same as in Fig. 1. Qualitatively the same results were obtained for HSA (not shown).

for the tendency of SA–BR mixtures 'supersaturated' in BR to restore 1 : 1 association on ageing along with the concomitant decrease in the fraction of retitratable BR.

If the same titrations are followed by nephelometry solutions remain essentially optically clear up to *r ca.* 1.8 but then light scattering becomes detectable and increases with time or if further BR is added (Fig. 3). This conforms with the above considerations that a fraction of BR not bound to SA forms a sol like system undergoing ageing¶ by coagulation and flocculation of solid BR. Since the size of particles, especially those of freshly prepared BR sols is still small the dispersed state can be detected by the naked eye only with some difficulty except when aligned perpendicular to transmittant light and may therefore easily escape attention.

An estimate of the contribution from BSA and HSA bound BR oligomers, respectively, to the overall CD spectrum at an excess of BR over SA was accomplished by subtracting the CD spectrum of the respective 1 : 1 complexes from those recorded at the largest ratio *r*. The two difference spectra obtained in this way (not shown) are very similar to each other showing CEs at λ *ca.* 440 nm (negative) and λ *ca.* 470 nm (positive). Therefore the smooth course of the CD monitored titration of HSA with BR if compared with the corresponding BSA titration (Fig. 1, see also ref. 12) mainly stems from the spectral similarity between the 1 : 1 associate and the contribution from multiple BR binding.

Adding the same portions of BR to PBS buffer in the absence of SA also affords a BR sol which undergoes more rapid ageing than is observed in the presence of SAs, as revealed by light scattering and UV–VIS measurements. This slightly different behaviour would conform with the general observation that a hydrophobic sol like BR becomes protected by the presence of hydrophilic organic additives. It seems to be these interactions, that underly the stabilising influence, which have been interpreted in terms of 'unspecific' BR binding.[1,9]

The fraction of titratable BR present in the pure sol decreases with time, to *ca.* 30% on immediate SA addition and to *ca.* 10% after 3 h. Further valuable information was obtained from this experiment. Firstly, light scattering becomes manifest at BR concentrations of *ca.* 1×10^{-6} mol dm^{-3} corresponding to *r ca.* 0.4 which apparently reflects the sensitivity of our method rather than the presence of a true solution since the solubility of BR at pH 7.4 is as low as 10^{-7}–10^{-8} mol dm^{-3}.[4,16] Hence it seems likely that flocculation of BR in the presence of SA actually begins at *r ca.* 1.4 rather than at $r = 1.8$ as is found directly (Fig. 3). Secondly, any BR sol prepared in this way is CD inactive in the BR absorption region which rules out the possibility that spectral changes as observed in the presence of SA during titration with BR (Fig. 1) are simply due to stray light. Thirdly, the inverse titration by adding SA to a preformed BR sol demonstrates the remarkable ability of SAs to redissolve and separate certain BR aggregates by tight binding in a 1 : 1 manner. Thus the relatively large, although still incomplete, degree of retitratable BR as found for freshly prepared SA–BR mixtures at large *r* ratios (see above) does not provide proof of the presence of monomers or even a large population of the SA : BR 1 : *n* ($n \geqslant 2$) complex. Finally, the position of the visible absorption band exhibited by the BR sol [λ *ca.* 440 nm (ε *ca.* 40 000 dm^3 mol^{-1} cm^{-1})] reflecting the property of aggregates is hypsochromically shifted by about 20 nm if compared with SA : BR 1 : 1 complexes (λ *ca.* 460 nm). Both the blue shift of this band observed during titration of SAs with BR beyond $r = 1$ and the red shift which takes place on ageing (Table 1) are in line with our above statements concerning the oligomeric state of BR if it is in excess over SA and agrees with observations delineated in ref. 9.

Conclusions

To facilitate the detection of SA : BR 1 : *n* complexes ($n > 1$) studies described in the literature[1,6,8–12] have commonly been carried out at an excess of BR over SA. The results obtained have then been extrapolated to *in vivo* conditions at which, however, the molar ratio *r* rarely exceeds unity even at lethal BR levels.[1,4,17] We believe that sequential BR binding to SA as often stated is only caused by these irregular conditions since SA–BR mixtures at $r > 1$ and $r \leqslant 1$ differ by more than their BR content. At $r > 1$ BR mainly adopts a metastable oligomeric state while at $r \leqslant 1$ the unbound BR is monomeric.[1,4,9] It is these oligomers which are involved in multiple binding onto SA but on aggregation proceeding more or less rapidly¶ they become successively withdrawn from the metastable equilibrium and the SA : BR 1 : *n* complex ($n \geqslant 2$) decomposes into its components. Our study therefore indicates that the maximum binding capacity of BSA and HSA, respectively, for BR is simply one as long as regular conditions, that is the range of true solutions, are employed. This is in line with recent binding studies[5] undertaken on SA–BR mixtures at $r = 1$ showing the absence of complexes with stoichiometries other than 1 : 1. The stability of BR oligomers along with the dispersed state critically depends on pH in agreement with ref. 9, but this observation does not provide a suitable explanation for the increasing risk of kernicterus at lowered plasma pH, since conditions at which BR might form a disperse system are never met *in vivo*. Whether multiple binding proceeds through stacking of BR at the primary site of

SA or if distinct 'secondary' sites for oligomeric BR species, inaccessible for the monomer, exist cannot be deduced from CD measurements alone. However, in view of the artificial conditions at which SA–BR 1 : *n* complexes ($n \geqslant 2$) occur this question would really be academic as already suspected[1] since an answer would hardly contribute to a better understanding of *in vivo* SA–BR interactions. Overall, our study favours the opinion that even at pathophysiological BR levels multiple BR binding onto SA is unimportant.

Experimental

General

BR (Serva) was chromatographed on silica gel prior to use. Serum albumins [BSA (97–99%, A 4378), BSA (fatty acid free, A 7511 and A 0281) 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. Palmitic acid (puriss. Fluka) was used without further purification. 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) or in phosphate buffer (PB) alone at pH 7.40. Bidistilled water was used throughout this study. For studies of pH dependent spectral changes the pH was adjusted (±0.05 units) to pH 7.1 with 1 mol dm^{-3} HCl using a Metrohm 691 pH meter. The glass electrode was calibrated by means of two buffer solutions (Metrohm). Prior to use buffers were sonicated for 15 min and then flushed with argon. Solutions of BR with 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 of variable path length (0.1–1 cm) at 20 ± 1 °C. Each spectrum is the average of 2–5 consecutive scans. Base line corrections between λ 350–600 nm were provided by buffer solutions. The SAs were CD inactive within this wavelength region. UV–VIS spectra were recorded with a Perkin-Elmer Lambda 7 instrument. Centrifugation was performed with a Sorvall RT 6000 B refrigerated centrifuge operating at *ca.* 3500 rpm and 15 °C. Light scattering within the range λ 550–700 nm was measured with a Perkin-Elmer Luminescence Spectrometer LS50B in 1 cm quartz cuvettes at ambient temperatures by synchronous scanning of the excitation and emission monochromator. Light scattering in Fig. 3 refers to λ 560 nm. Baselines were obtained from solutions before addition of BR. All measurements described below were run at least in triplicate. For the determination of free sulfhydryl groups of SAs [0.60 and 0.18 SH per molecule for BSA (A 4378) and HSA (A 3782), respectively] see ref. 5.

Titration of HSA and BSA with BR

Solutions A–C were prepared as follows. Solution A. BR (0.59 mg, 1.0×10^{-6} mol) was rapidly dissolved in 0.025 M KOH (290 mm^3) followed by the addition of the appropriate buffer (1170 mm^3). 58 mm^3 of this solution contains 4×10^{-8} mol BR. This solution is stable for at least 30 min at 0 °C. Solution B. The corresponding SA (53.2 mg, 8×10^{-7} mol) was dissolved in buffer and adjusted to 20 ml. Solution C. The corresponding SA (200 mg, 3.0×10^{-6} mol) was dissolved in buffer and adjusted to 5 ml.

Continuous titrations. Solution B (0.25 ml, containing 1×10^{-8} mol SA) and 2.25 ml buffer were transferred into a 1 cm quartz cuvette equipped with a magnetic stirrer and titrated by successive addition of solution A (14.5 mm^3 refer to 1 equiv. BR) followed by CD and UV–VIS measurements. Light scattering measurements were obtained from separate experiments. During all titrations the pH was monitored by pH measurements directly performed in the cuvette using a glass electrode.

Discontinuous titrations. This mode was used for ageing and retitration experiments. Solution B (1 cm^3, 4×10^{-8} mol) was injected into several 10 ml flasks each containing the appropriate buffer (*ca.* 7 ml). Then various amounts of solution A (58 mm^3 refer to 1 equiv. BR) were added to each flask and filled up to 10 ml with the appropriate buffer to obtain solutions of the desired *r* ratios. 2.5 ml of these solutions were immediately subjected to spectroscopic measurements. Retitrations with the corresponding SA were performed by addition of solution C (16.4 mm^3 refer to 1×10^{-8} mol SA).

The course of titrations obtained for undefatted BSA, defatted BSA and defatted BSA preloaded with two equivs. of palmitic acid was essentially the same.

Successive addition of BR to PBS buffer

2.5 ml of PBS buffer was transferred into a 1 cm cuvette followed by successive addition of solution A as described for the continuous titration mode, and then subjected to spectroscopic measurements.

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