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Inversion of Circular Dichroism with a Drop of Chloroform. Unusual Chiroptical Properties of Aqueous Bilirubin–Albumin Solutions

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Abstract: The magnitude and sign of the characteristic circular dichroism spectrum of bilirubin bound to human serum albumin are highly sensitive to the presence of trace amounts of dissolved chloroform. The presence of ~40 mM chloroform in an aqueous solution containing 0.25 mM bilirubin and 0.44 mM albumin is sufficient to cause complete inversion of the circular dichroism curve obtained without chloroform. Addition of chloroform to the albumin solution before or after the bilirubin has the same effect. The sign inversion is not due to a chemical reaction because it was reversed completely on removal of chloroform, regenerating curves characteristic of chloroform-free solutions. In contrast to its effect on the pigment circular dichroism, chloroform had relatively little effect on the circular dichroism of the albumin. These observations show that reversible solvation of chloroform within albumin can alter the conformation and exciton chirality circular dichroism of the bound ligand, probably by changing the internal topography or dynamics of the bilirubin binding site.

Introduction

Bilirubin, the cytotoxic yellow orange pigment of jaundice and the major linear tetrapyrrole product of mammalian heme metabolism, forms a high affinity association complex with human serum albumin (HSA) and other proteins involved in its transport and excretion.[1-4] The association constants (K_A) for the bilirubin–HSA complex ($K_A \simeq 10^8$ M^{-1} for the first bilirubin molecule and $K_A \simeq 10^5$ M^{-1} for a second bilirubin) have been determined by analyzing spectral shifts in the UV–visible absorption spectrum and the circular dichroism (CD) spectrum and confirmed independently by other methods.[1-3] In water at physiologic pH, the bilirubin–HSA complex exhibits a broad UV–visible absorption band near 460 nm and an intense bisignate, almost sinusoidal CD spectrum.[1]

In addition to its use as an analytical tool for assessing quantitative aspects of bilirubin–protein binding, CD is known to be a powerful spectroscopic probe of stereochemistry.[5,6] For bilirubin–protein complexes the typically intense bisignate CD centered near 430 nm[1,7-10] originates from exciton coupling[5,11] of the pigment's two dipyrrinone chromophores held in a chiral orientation by the protein.[7-9,12,13] Since the signed order and intensity

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(1) McDonagh, A. F. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 6, pp 293–491.
(2) Brodersen, R. In *Bilirubin*; Heirwegh, K. P. M., Brown, S. B., Eds.; CRC Press: Boca Raton, FL, 1982; Vol. 1, pp 75–123.
(3) For leading references, see: Ostrow, J. D., Ed. *Bile Pigments and Jaundice*; Marcel Dekker, Inc.: New York, 1986.
(4) Lightner, D. A.; McDonagh, A. F. *Acc. Chem. Res.* **1984**, *17*, 417–424.
(5) Harada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy. Exciton Coupling in Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983.
(6) Deutsche, C. W.; Lightner, D. A.; Woody, R. W.; Moscowitz, A. *Annu. Rev. Phys. Chem.* **1969**, *20*, 407–448.
(7) For leading references, see: Blauer, G. *Isr. J. Chem.* **1983**, *23*, 201–209.
(8) Lightner, D. A.; Wijekoon, W. M. D.; Zhang, M.-H. *J. Biol. Chem.* **1988**, *263*, 16669–16676.
(9) Lightner, D. A.; Reisinger, M.; Landen, G. L. *J. Biol. Chem.* **1986**, *261*, 6034–6038.
(10) Blauer, G. *Biochem. Biophys. Acta* **1988**, *884*, 602–604.
(11) Lightner, D. A.; Gawroński, J. K.; Wijekoon, W. M. D. *J. Am. Chem. Soc.* **1987**, *109*, 6354–6362.

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Table I. Inversion of Circular Dichroism (CD) Spectra of Aqueous Bilirubin-Human Serum Albumin (BR-HSA) Solutions before and after Extraction with Chloroform[a]

		[BR]:[HSA] = 1:1.75[b,c]				[BR]:[HSA] = 2:1[b,d]			
		circular dichroism[e]			UV-vis[e]	circular dichroism[e]			UV-vis[e]
pH	extraction	$\Delta\epsilon_{max}(\lambda_1)$[g]	λ at $\Delta\epsilon = 0$	$\Delta\epsilon_{max}(\lambda_3)$[g]	$\epsilon_{max}(\lambda)$[g]	$\Delta\epsilon_{max}(\lambda_1)$[g]	λ at $\Delta\epsilon = 0$	$\Delta\epsilon_{max}(\lambda_3)$[g]	$\epsilon_{max}(\lambda)$[g]
9.1	before	+30 (453)	425	-27 (404)	45 000 (460)	+47 (452)	427	-40 (406)	47 000 (458)
	after[f]	-130 (457)	427	+83 (406)	53 000 (454)	-106 (457)	427	+68 (406)	52 000 (455)
7.0	before	+38 (462)	427	-21 (407)	42 000 (455)	+44 (457)	427	-34 (403)	42 500 (455)
	after[f]	-90 (465)	433	+53 (417)	50 000 (455)	-84 (460)	429	+50 (406)	49 000 (452)
6.0	before	+40 (452)	416	-15 (400)	41 500 (450)	+24 (453)	418	-7 (407)	42 000 (452)
	after[f]	-27 (458)	428	+17 (407)	46 000 (452)	-15 (465)	427	+9 (417)	40 000 (452)
4.5	before	-13 (466)	438	+10 (418)	37 400 (452)				
	after[f]	-13 (466)	437	+11 (417)	37 800 (453)				

[a] [BR] = [HSA] = 2.2×10^{-4} M. At pH = 8.0 and a [BR]:[HSA] molar ratio of 1:1: before extraction, $\Delta\epsilon(\lambda_1{=}452) = +68$, $\lambda_2 = 426$ at $\Delta\epsilon = 0$ and $\Delta\epsilon(\lambda_3{=}403) = -43$; after extraction, $\Delta\epsilon(\lambda_1{=}457) = -102$, $\lambda_2 = 426$ at $\Delta\epsilon = 0$, and $\Delta\epsilon(\lambda_3{=}406) = +63$. [b] [BR] = 2.50×10^{-4} M. [c] [HSA] = 4.41×10^{-4} M. [d] [HSA] = 0.125×10^{-4} M. [e] ϵ and $\Delta\epsilon$ in $L \cdot mol^{-1} \cdot cm^{-1}$ and λ in nm. [f] ϵ and $\Delta\epsilon$ corrected for pigment loss into chloroform. [g] λ values in parentheses.

of the CD Cotton effects depends crucially on the *relative orientation* of the two dipyrrinone halves,[11,12,14] CD can be used to probe the stereochemistry of bound bilirubin and the topography of the protein binding site.

Most serum albumins bind bilirubin and induce CD, sometimes with a positively signed but usually with a negatively signed longer wavelength transition of the bisignate exciton couplet.[7] HSA has repeatedly been shown to induce a long wavelength positive, short wavelength negative bisignate CD for bilirubin in aqueous solution at pH >6, over a wide range of HSA:bilirubin molar ratios ranging from 15:1 to 1:2.[15] At pH 7.4 and a 2:1 ratio, typical CD values are $\Delta\epsilon_{460}^{max} \simeq +49$ M^{-1} cm^{-1} and $\Delta\epsilon_{407}^{max} \simeq -26$ M^{-1} cm^{-1}. In contrast, at pH 4.05 and a 15:1 ratio, the bisignate curve is inverted and shifted bathochromically, with values as large as $\Delta\epsilon_{473}^{max} = -214$ M^{-1} cm^{-1} and $\Delta\epsilon_{423}^{max} = +130$ M^{-1} cm^{-1}. The origin of the pH-induced CD change is thought to be associated with a change in secondary structure of the albumin.[15]

We recently noted that volatile anesthetics cause an inversion of the characteristic CD of bilirubin-HSA complex, apparently reflecting a major conformational change of the bound chromophore.[16] In the current work we describe more extensive studies on the effects of chloroform on the structure of bilirubin complexed with albumin.

Experimental Methods

Bilirubin (Sigma Chemical Co.) contained less than 5% of the IIIα and XIIIα isomers, as determined by high-performance liquid chromatography.[17] For purposes of UV and CD spectral measurements the molecular weight of HSA was taken to be 68 000. Human serum albumin (HSA), both defatted and undefatted (Sigma), was used as obtained. The CD results were essentially the same with both. The organic solvents used were spectral grade. A pH 8.0 solution of bilirubin-HSA, 2.2×10^{-4} M in bilirubin and HSA, was prepared by dissolving 1.28 mg (2.20×10^{-3} mmol) of bilirubin in 0.2 mL of 0.1 M KOH and adding this solution to a solution of 150 mg (2.20×10^{-3} mmol) of HSA in 9.5 mL of distilled water. The pH was adjusted to 8.0 by the addition of 0.1 M aqueous KOH, and the total volume was brought to 10 mL by addition of water. Solutions 2.5×10^{-4} M in bilirubin with different mole ratios of HSA and specified pH were prepared as follows: (i) A pH 7.0 solution of bilirubin:HSA (1:1.75 mole ratio) was prepared by dissolving 300 mg (4.41×10^{-3} mmol) of HSA in 9.5 mL of distilled water followed by addition of a solution of 1.46 mg (2.50×10^{-3} mmol) of bilirubin in 0.20 mL of 0.1 M aqueous KOH and adjustment of the volume to 10 mL with water. (ii) Solutions of pH 9.0, 6.0, and 4.5 with a bilirubin:HSA molar ratio of 1:1.75 were prepared as in (i) with the pH being adjusted by the addition of 0.1 M aqueous KOH or 0.1 M aqueous HCl. (iii) A pH 7.0 solution of bilirubin:HSA, molar ratio 2:1, was prepared as in (i) except 85.5 mg (1.25×10^{-3} mmol) of HSA was used. (iv) Solutions of pH 9.0, 6.0, and 4.5 with a bilirubin:HSA molar ratio of 2:1 were prepared as in (iii) with the pH being adjusted by the addition of 0.1 M aqueous KOH or 0.1 M aqueous HCl. All solutions were equilibrated for 2 h in the dark at 5 °C and then brought to room temperature for CD studies. CD spectra were recorded on a JASCO J-600 spectropolarimeter; UV-visible spectra were run on a Cary 219 spectrophotometer.

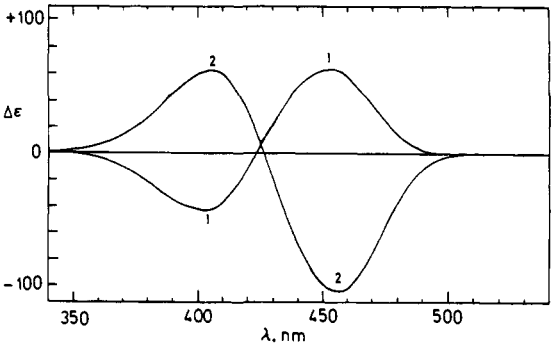

Figure 1. Circular dichroism spectrum of an aqueous solution of 2.20×10^{-4} M bilirubin and 2.20×10^{-4} M HSA at pH 8.0 and 20 °C (curve 1). CD spectrum of the same solution after one extraction with an equal volume of chloroform and correction for loss of pigment due to extraction (curve 2). Aspiration of the latter solution to remove chloroform regenerated the original CD spectrum. The $\Delta\epsilon = 0$ line corresponds to the CD spectrum of bilirubin in the absence of HSA.

Results and Discussion

The CD spectrum of a pH 8.0 solution with equimolar (1:1) bilirubin and HSA (2.20×10^{-4} M in each) showed the characteristic bisignate shape,[7-9] with a long wavelength positive, short wavelength negative exciton couplet (Figure 1). When the solution was washed once with an equal volume of chloroform by gentle inversion in a separatory funnel, about 10% of the bilirubin partitioned into the organic phase. Astonishingly, the CD spectrum of the aqueous phase was found to be inverted, and the intensity (after correction for pigment loss) was increased (Figure 1). Brief aspiration of the aqueous phase on a rotary evaporator (water aspirator) at room temperature to remove dissolved chloroform caused the CD signs to revert back to those of the original solution, and the CD Cotton effects showed 81-93% of the original intensity. The sign inversion phenomenon was not unique to a particular molar ratio of bilirubin:HSA and also occurred over a wide pH range. For example, reversible sign inversions could be induced from pH 6.0-9.1 over a range of bilirubin:HSA molar ratios varying from 1:1.75 to 2:1 (Table I).

The CD sign inversions illustrated in Figure 1 can be shown to depend on the presence of chloroform rather than extraction of pigment by chloroform. Thus, when 10-μL aliquots of chloroform were added successively to a solution containing equimolar bilirubin and HSA (2.20×10^{-4} M in each, prepared as above), the CD intensities decreased and the signs inverted to give a set of CD curves with a tight isosbestic point near 425 nm (Figure

(12) Blauer, G.; Wagnière, G. *J. Am. Chem. Soc.* **1975**, *97*, 1949-1954.
(13) Lauffer, R. B.; Vincent, A. C.; Padinanabhan, S.; Meade, T. J. *J. Am. Chem. Soc.* **1987**, *109*, 2216-2218.
(14) Lightner, D. A.; Person, R. V.; Peterson, B. R.; Puzicha, G.; Pu, Y.-M.; Bojadziev, S. *Biomolecular Spectroscopy II*; Birge, R. R., Nafie, L. A., Eds.; Proc. SPIE 1432, 1991; pp 2-13.
(15) Blauer, G.; Harmatz, D.; Snir, J. *Biochem. Biophys. Acta* **1972**, *278*, 68-88.
(16) For leading references see: McDonagh, A. F.; Pu, Y. M.; Lightner, D. A. *Experientia* **1992**, *48*, 246-248.
(17) McDonagh, A. F.; Palma, L. A.; Trull, F. R.; Lightner, D. A. *J. Am. Chem. Soc.* **1982**, *104*, 6865-6867.

Table II. Influence of Adding 10-μL Aliquots of Chloroform on the CD Spectrum[a] of Aqueous Solutions (10 mL) of Bilirubin and HSA

added $CHCl_3$		[BR]:[HSA][b] = 1:1.75, pH = 7.0			[BR]:[HSA][c] = 2:1, pH = 7.0			[BR]:[HSA][d] = 1:1, pH = 8.0		
aliquot	total, mmol	$[CHCl_3]$: [HSA]	$\Delta\epsilon_{max}(\lambda_1)$[e]	$\Delta\epsilon_{max}(\lambda_2)$[e]	$[CHCl_3]$: [HSA]	$\Delta\epsilon_{max}(\lambda_1)$[e]	$\Delta\epsilon_{max}(\lambda_2)$[e]	$[CHCl_3]$: [HSA]	$\Delta\epsilon_{max}(\lambda_1)$[e]	$\Delta\epsilon_{max}(\lambda_2)$[e]
0	0	0:1	+38 (462)	−21 (407)	0:1	+44 (457)	−34 (403)	0:1	+68 (452)	−43 (404)
1	0.125	28:1	+21 (460)	−10 (403)	100:1	+27 (454)	−19 (404)	57:1	+27 (451)	−21 (402)
2	0.250	57:1	−6 (465)	+6 (415)	200:1	+14 (454)	−12 (404)	114:1	+8 (448)	−11 (400)
3	0.375	85:1	−34 (458)	+18 (407)	300:1	≪0.1 (454)	−5 (404)	170:1	−44 (456)	+20 (407)
4	0.500	113:1	−50 (458)	+28 (407)	400:1	−11 (454)	+2 (404)	227:1	−68 (456)	+41 (407)
5	0.625	142:1	−62 (457)	+35 (407)	500:1	−18 (454)	+6 (404)	285:1	−100 (457)	+57 (407)
6	0.750				600:1	−25 (454)	+12 (404)			
7	0.875				700:1	−30 (454)	+15 (404)			

[a] $\Delta\epsilon$ in $L \cdot mol^{-1} \cdot cm^{-1}$ and λ in nm. [b] 4.41×10^{-4} M HSA. [c] 1.25×10^{-4} M HSA. [d] 2.2×10^{-4} M HSA. [e] λ values in parentheses.

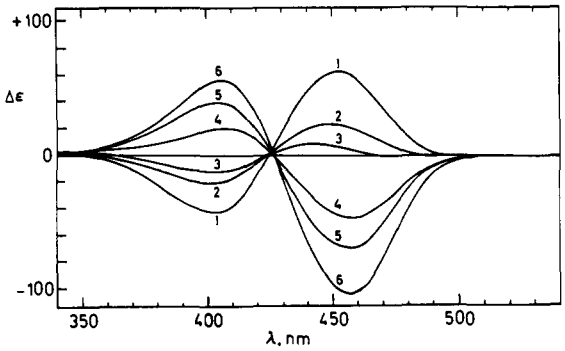

Figure 2. Circular dichroism spectra of an aqueous solution (3 mL) of 2.20×10^{-4} M bilirubin and 2.20×10^{-4} M HSA at pH 8.0 and 20 °C measured before (curve 1) and after the successive addition of 10 μL aliquots of chloroform: 10 μL total (curve 2), 20 μL total (curve 3), 30 μL total (curve 4), 40 μL total (curve 5), and 50 μL total (curve 6). The $\Delta\epsilon = 0$ line corresponds to the CD spectrum of bilirubin in the absence of HSA. The tight isosbestic point implies a smooth interconversion between two molecular species.

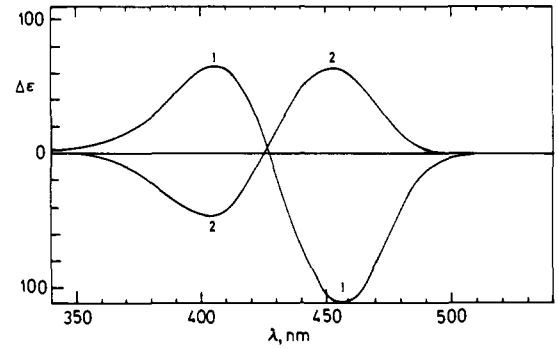

Figure 3. Circular dichroism spectrum of a pH 8.0 aqueous solution (3 mL) of 2.20×10^{-4} M bilirubin and 2.20×10^{-4} M HSA at 20 °C prepared by addition of 50 μL of chloroform to the HSA solution before addition of bilirubin (curve 1). Aspiration of the CD solution to remove chloroform returns the CD spectrum to near normal (curve 2). The $\Delta\epsilon = 0$ line corresponds to the CD spectrum of bilirubin in the absence of HSA.

2). These large changes were accompanied by only minor changes in the UV–visible absorption spectrum. After the addition of a total of 50 μL of chloroform, which would be saturation for pure water, the CD spectrum was nearly the same as that measured following extraction with chloroform (above). And as before, the CD spectra reverted to their original signs and magnitudes on aspiration (suction) to remove dissolved chloroform (as in curve 6 → curve 1 of Figure 2).

Since CD spectroscopy in the far-ultraviolet region has been used widely and successfully to detect and analyze changes in protein conformation, we measured the CD of aqueous HSA solutions before the addition of chloroform and after saturation with chloroform. Only very minor changes were detected. These results indicate that no large changes in the ordered structure of serum albumin occur within the pH range investigated (pH 6–9) following the addition of chloroform. Thus, the bilirubin–HSA CD sign inversion observed upon addition of chloroform probably reflects subtle changes in the binding site microenvironment rather than a large change in protein conformation such as the N→F transition seen at pH values near 4.5.[18,19]

When chloroform (50 μL) was added to the aqueous HSA solution *before* adding bilirubin, the CD spectrum of the bilirubin–HSA complex was of the inverted type (Figure 3), and again the CD spectrum returned to its normal shape on removal of the chloroform.

CD inversions on addition of chloroform were seen for solutions containing 1:1.75 and 2:1 molar ratios of bilirubin:HSA (Table II) and at pH 9.1, 7.0, and 6.0. At pH 6, however, the magnitudes of the inverted CD curves did not exceed the original values, as for solutions at the higher pH values. At pH 4.5 the CD signs are inverted even in the absence of chloroform, as has been noted previously,[7,15] and they remain inverted and unchanged upon addition of chloroform.

As noted previously, other low molecular weight, water-insoluble halogenated hydrocarbons also induce CD sign inversions.[16] For example, extraction with dichloromethane has the same effect as chloroform extraction. In contrast, carbon tetrachloride extraction leads only to reduced intensities ($\Delta\epsilon_{460}^{max}$ +7.8, $\Delta\epsilon_{410}^{max}$ +7.0 M^{-1} cm^{-1}), with less bilirubin being extracted. Extraction with benzene or *n*-hexane resulted in apparent protein denaturation and complete loss of CD (hence optical activity) from bilirubin.

Cotton effect sign inversions of the bilirubin–HSA complex have been recognized previously under special circumstances—for example, near pH 4[1] where it is thought that the protein undergoes a change in secondary structure.[2] High concentrations of added low molecular weight alcohols, particularly primary alcohols, e.g. 1-pentanol, also invert the CD.[20] However, the sensitivity of the CD spectrum to added halogenated hydrocarbons, especially chloroform, dichloromethane, and halothane,[16] is particularly striking because relatively low concentrations exert a profound effect, inverting the Cotton effects and enhancing their magnitudes (Table II). The Cotton effect signs become inverted at a $CHCl_3$:HSA molar ratio of ~57:1 for the 2:1 HSA:bilirubin complex at pH 7.0, and marked diminution of the CD can be detected at much lower $CHCl_3$:HSA ratios. In contrast, with 1-pentanol an alcohol:HSA molar ratio of ~500:1 is required to effect sign inversion of the CD spectrum of the 2:1 HSA:bilirubin complex and molar ratios of ~350:1 are required to effect major changes in the CD spectrum. Chloroform appears to be an order of magnitude more effective than organic alcohols. Other molecules which compete with bilirubin for a common binding site, e.g., certain fatty acids,[7,21,22] salicylate,[23] and penicillin,[23] may lead to changes in the CD of the bilirubin–albumin complex, but the effects are not nearly as intense as those seen with chloroform

(18) Foster, J. F. In *The Plasma Proteins*; Putham, F. W., Ed.; Academic Press: New York, 1960; pp 179–239.
(19) Brodersen, R. *CRC Crit. Rev. Clin. Lab. Sci.* **1980**, *11*, 305–399.
(20) Blauer, G.; Lavie, E. In *Protein–Ligand Interactions*; Sund, H., Blauer, G., Eds.; Walker de Gruyter: New York, 1975; pp 399–411.
(21) Blauer, G.; Harmatz, D. *Biochim. Biophys. Acta* **1972**, *278*, 89–100.
(22) Woolley, P. V., III; Hunter, M. J. *Arch. Biochem. Biophys.* **1970**, *140*, 197–209.
(23) Lavie, E.; Blauer, G. *Arch. Biochem. Biophys.* **1979**, *193*, 191–203.

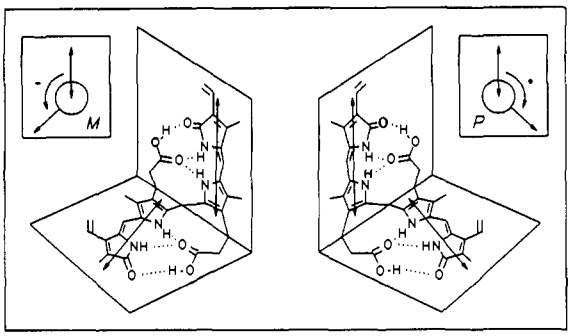

Figure 4. Interconverting intramolecularly hydrogen bonded enantiomeric conformers of bilirubin-IXα. The double headed arrows represent the dipyrrinone long wavelength electric transition moment vectors (dipoles). The relative helicities (*M*, minus; *P*, plus) of the vectors are shown (inset) for each enantiomer.

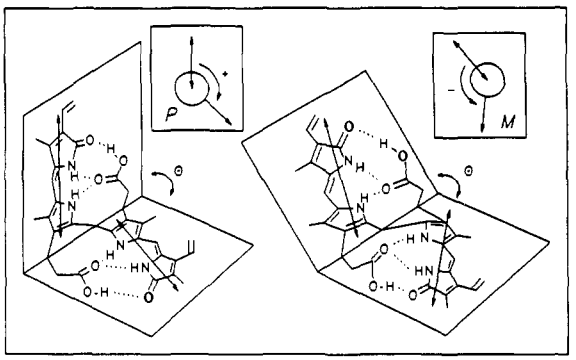

Figure 5. (Left) *P*-Chirality bilirubin in its intramolecularly hydrogen bonded ridge-tile conformation. The intersection of the two dipyrrinone planes makes a dihedral angle, $\theta \simeq 100°$. (Right) The same conformation with a larger θ and a flatter ridge-tile shape. Flattening is accommodated by lengthening (or breaking) hydrogen bonds between the carboxylic acid CO_2H and lactam -NH-C=O groups. More significantly, the transition dipole vectors associated with the dipyrrinone long wavelength UV-visible absorption reverse relative orientation (from *P* to *M*).

and are not readily reversible.[19] Interestingly, the concentration of chloroform that causes CD sign inversion is of the same order of magnitude as the concentration of (+)-isoflurane required to inhibit ion channel currents in isolated molluscan neurons.[24]

The intense CD of the bilirubin-HSA complex originates from the chirality of the tetrapyrrole ligand.[8] Although the structure of bilirubin bound to HSA is not known, chiral ridge-tile conformations (Figure 4) similar to those observed in crystalline bilirubin[6,25] are likely. As shown by CD, binding is enantioselective, favoring a bilirubin conformation with ***P*** (plus), rather than ***M*** (minus) chirality.[8] The sigmoidal shape of the CD spectrum reflects exciton coupling[5] between the two dipyrrinone chromophores of the ligand. The signed order of the two exciton components depends on the relative helical orientation (***P*** or ***M***) of the electric transition dipole vectors associated with each chromophore and the relative order of the exciton excited states.[11] Inversion of the bilirubin-HSA CD spectrum in the presence of chloroform must be caused by a change in the stereochemistry of the ligand presumably brought about by nonspecific co-binding of chloroform to the protein. This change in stereochemistry must in turn lead to an inversion of the relative helicity (*P*→*M*) of the transition dipole vectors or inversion of the relative order of the exciton energy levels. It is unlikely that the CD sign inversion simply reflects displacement of bilirubin from a primary to a secondary binding site with different topography because the CD of bilirubin bound to secondary sites has the same signed order as that of bilirubin bound to the primary site, and because the CD sign inversion occurs at bilirubin:HSA molar ratios of 1:2. The precise nature of the stereochemical change leading to the sign inversion is unclear. However, it must be large enough to reverse the helicity of the coupled electric transition dipole vectors or at least sufficient to invert the relative energies of the exciton levels.[27] Molecular modeling studies[14,28] show that helical inversion can be brought about either by complete enantiomerism, as shown in Figure 4, or by a flattening of the ridge-tile conformation of bilirubin, as shown in Figure 5. Enantiomeric inversion to a mirror image form within the protein binding site seems unlikely. Flattening of the ridge-tile conformation seems more plausible, and precedence for this explanation may be found in the recently observed solvent-induced CD sign inversion of a bis-dipyrrinone model compound.[27a] It is important to recognize that inversion of exciton CD Cotton effects may occur without an inversion of molecular chirality. *Therefore oppositely signed CD curves do not necessarily reflect mirror image structures.*

Numerous studies have been reported on CD spectra of bilirubin-HSA complexes, but relatively little is known about the CD of photoisomers of bilirubin which are important in phototherapy of neonatal jaundice.[29] Lamola et al.[30] have reported on the CD of photobilirubin bound to HSA. The aqueous solutions used in those studies probably contained chloroform originating from an extraction step in their preparation, which may have influenced the observed CD spectra. Presently we do not know whether the CD spectra of photoisomers of bilirubin are as sensitive to halocarbons as the parent isomer. However, our results do indicate a potential unrecognized potential danger in using CD spectroscopy to monitor changes in bilirubin biochemistry or photochemistry when chloroform or dichloromethane extractions are used in the preparation of solutions.

Summary

Millimolar concentrations of chloroform and other volatile anesthetics completely invert and even magnify the intensity of the characteristic CD spectrum of pH 6–9 aqueous bilirubin-HSA solutions. The spectral sign inversion, which is completely reversed by removal of the chloroform, reflects a pronounced conformational change of the bound ligand. Our observations suggest that nonspecific association of halocarbons with proteins or the dissolution of halocarbons within the hydrophobic domains of proteins can markedly alter the microenvironment and internal topography of specific binding sites.

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Registry No. $CHCl_3$, 67-66-3.

(24) Franks, N. P.; Lieb, W. R. *Science* **1991**, *254*, 427–430.
(25) Bonnett, R.; Davies, J. E.; Hursthouse, M. B.; Sheldrick, G. M. *Proc. R. Soc. London* **1978**, *B202*, 249–268.
(26) Le Bas, G.; Allegret, A.; Mauguen, Y.; De Rango, C.; Bailly, M. *Acta Crystallogr., Sect. B* **1980**, *B36*, 3007–3011.
(27) These are possible explanations for the results observed in: (a) Byun, Y. S.; Lightner, D. A. *J. Org. Chem.* **1991**, *56*, 6027–6033. (b) Gargiulio, D.; Derguini, F.; Berova, N.; Nakanishi, K.; Harada, N. *J. Am. Chem. Soc.* **1991**, *113*, 7046–7047.
(28) Person, R. V.; Boiadjiev, S. E.; Peterson, B. R.; Puzicha, G.; Lightner, D. A. 4th International Conference on Circular Dichroism, Sept. 9–13, Bochum, FRG, 1991; pp 55–74.
(29) McDonagh, A. F.; Lightner, D. A. *Pediatrics* **1985**, *75*, 443–455.
(30) Lamola, A. A.; Flores, J.; Doleiden, F. H. *Photochem. Photobiol.* **1982**, *35*, 649–654.