Conformation of Bilirubin and Biliverdin in Their Complexes with Serum Albumin

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Abstract: The 1:1 complexes of bilirubin and biliverdin with human serum albumin are of physiological importance. The long-wavelength absorption and CD spectra in aqueous solution indicate a highly specific mode of binding between the pigment and the protein. It has been reported that the observed long-wavelength Cotton effects show rotatory strengths of the order of 1–6 $D\mu_B$ (Debye–Bohr magnetons). A change of pH from 4 to 10 causes an inversion in the sign of these Cotton effects. Conformational or other changes in the protein apparently invert the chirality of the bound pigment. In this work we report on the chiroptic properties of the pigment chromophores as a function of two characteristic dihedral angles, δ_1 and δ_2, using a MO–SCF–CI procedure in the frame of an adapted PPP approximation. Electric and magnetic transition moments were computed without further approximations. From the calculated dipole and rotatory strengths we conclude that at neutral or higher pH the dipyrromethene moieties in the bilirubin complex should be rotated with respect to each other in a right-handed conformation, for which $(\delta_1 + \delta_2) \simeq +30°$. At low pH, it is estimated that $(\delta_1 + \delta_2) \simeq -50°$. There is tentative agreement only between calculations and experimental data for the biliverdin complexes, and further possible conformations of bound biliverdin will have to be taken into account.

The physiological importance of complexes of serum albumin with the bile pigments bilirubin (BR) and biliverdin (BV) is well known (e.g., ref 1). In view of the specific binding of these pigments to the protein, the optical absorption and CD spectra of the complexes in aqueous solution, in particular of those with human serum albumin (HSA), have been thoroughly investigated.[2-7]

In the light-absorption spectrum (Figure 1) of the complex BR–HSA there is a broad band between 400 and 500 nm which appears to be due to at least two transitions separated by roughly 50 nm (2500 cm^{-1}). The presence of two distinct electronic transitions is strikingly revealed in the CD spectra.[3,4,6,7] One finds (Figure 2) at low pH (4.0; charcoal-treated HSA) a strong negative CD band at 474 nm ($[\theta]_{max} \simeq -70 \times 10^4$), followed by a strong positive one at 422 nm ($[\theta]_{max} \simeq +40 \times 10^4$). ($[\theta]$ is the molar ellipticity in deg cm^2 $dmol^{-1}$, based on bile pigment; ϵ is the molar extinction coefficient in M^{-1} cm^{-1}, based on bile pigment.) When the pH is raised (7.3; 9.7) the sign of the Cotton effects is inverted, with some changes in wavelength of the band extrema, and the absolute $[\theta]_{max}$ values are one order of magnitude lower under the given experimental conditions. This leads us to suspect a specific change in the chirality of the bound pigment, influenced by the conformational changes of the protein.[3,4]

In biliverdin, in contrast to bilirubin, the two dipyrromethenes are conjugated. This relatively small structural difference in the center of the molecule induces a strong red shift of the longest wavelength absorption. Also, free rotation within the molecule is prevented. The visible spectrum (Figure 3) of the BV–HSA complex is thus characterized by a relatively broad absorption around 660–670 nm ($\epsilon_{max} \simeq 2 \times 10^4$), followed by the next band at 380–390 nm ($\epsilon_{max} \simeq 4 \times 10^4$).[5] In the CD spectrum (Figure 4) at neutral pH (7.4) the Cotton effect of the 380–400-nm band is positive ($[\theta]_{max} \simeq 25 \times 10^4$), and one finds the onset of a negative Cotton effect at 600 nm. Because of experimental limitations, the CD spectrum has been measured only to 650 nm, but from the shape of the curve, $[\theta]_{max}$ appears to be of the order of 10×10^4 in absolute magnitude.

Computations. The striking spectroscopic features mentioned above prompted us to draw several conclusions about the conformation of the pigments in complexes with albumin. For simplicity, we assume that the two individual dipyrromethene moieties of bilirubin in the complex remain undistorted and planar, and we characterize the pigment molecule by only two dihedral angles, δ_1 and δ_2, as shown in Figure 5a. For $(\delta_1 + \delta_2) > 0$, the bilirubin chromophore has a right-handed chirality, for $(\delta_1 + \delta_2) < 0$, a left-handed one. If one knows not only the magnitude but also the direction of polarization of the longest wavelength band in dipyrromethene (Figure 6), one is tempted to predict the long-wavelength absorption and CD spectrum of the composite bilirubin system by the coupled oscillator model.[3,8] However, this presupposes that the individual dipyrromethene transition moments may be appropriately localized within the monomers. When the dimensions of the individual monomers become comparable to their separation, the applicability of the coupled oscillator model in its simplest form may be questioned.[9,10] We therefore have chosen, instead, to consider the bilirubin chromophore as an entity (Figure 7) and, assuming local σ–π separation within the dipyrromethene moieties, to apply a suitable SCF–MO–CI procedure in the general frame of the PPP approximation.[11] Although resonance integrals β_{pq} were considered only between nearest neighbors within the individual dipyrromethene halves, the electron repulsion integrals γ_{pq} between all 24 atomic centers of the composite system entered the calculation. The parametrization (see Tables I and II) was identical with the one already successfully used to interpret the optical spectra of aromatic compounds containing the heteroatoms N and O.[12] The interaction of the 100 lowest singly excited configurations was taken into account.

From these semiempirical wave functions we have calculated oscillator strengths and rotatory strengths without further approximations. The electric transition moments, in the dipole velocity form, and the magnetic transition moments were computed as described previously.[13,14] For all conformations δ_1/δ_2, the axis of every $2p_\pi$ atomic orbital was taken to be perpendicular to the plane of the dipyrromethene moiety to which the atom in question belongs.

Biliverdin has, of course, 25 centers of unsaturation. As its π-electron system is isoelectronic with that of bilirubin, one nitrogen atom will be of the "pyridine-type." For simplicity, we characterize the conformations of biliverdin by the same two dihedral angles, δ_1 and δ_2. The resonance integrals of the corresponding bonds, β_{2-25} and β_{25-14} (Figure 7), are then to be multiplied by a factor $\cos|\delta_1|$ and $\cos|\delta_2|$,

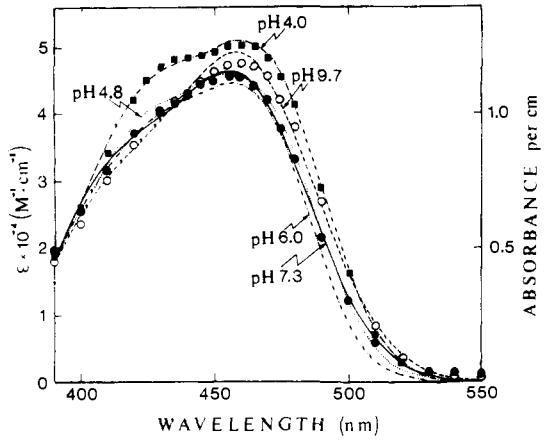

Figure 1. Long-wavelength absorption spectrum of the BR–HSA complex at different pH values. (Reproduced from ref 3, where details are given.)

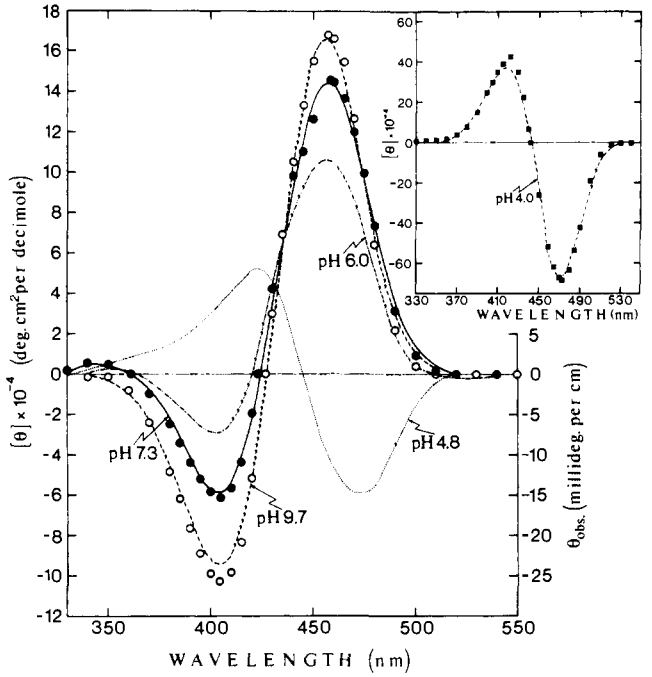

Figure 2. Long-wavelength CD spectrum of the BR–HSA complex. Note the pH dependence. (Reproduced from ref 3, where details are given.)

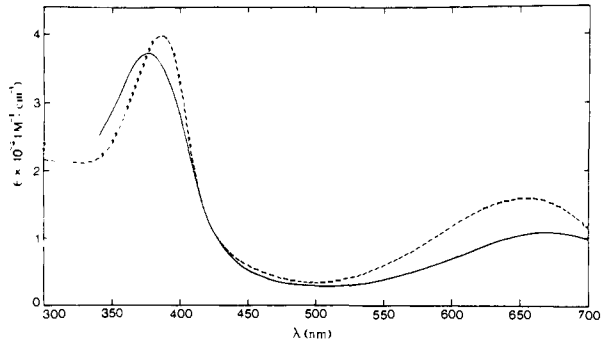

Figure 3. Long-wavelength absorption spectrum of free biliverdin at pH 11.8 (full line) and of the BV–HSA complex at pH 7.4 (dotted line). (Reproduced from ref 5, where details are given.)

respectively. We assume that the loss of resonance energy incurred by twisting the molecule out of the plane may be outweighed by intermolecular interactions of the pigment with the protein. Obviously, much higher energies are required to produce the above-mentioned biliverdin conformations as compared with bilirubin which has simple carbon–carbon bonds in its center.

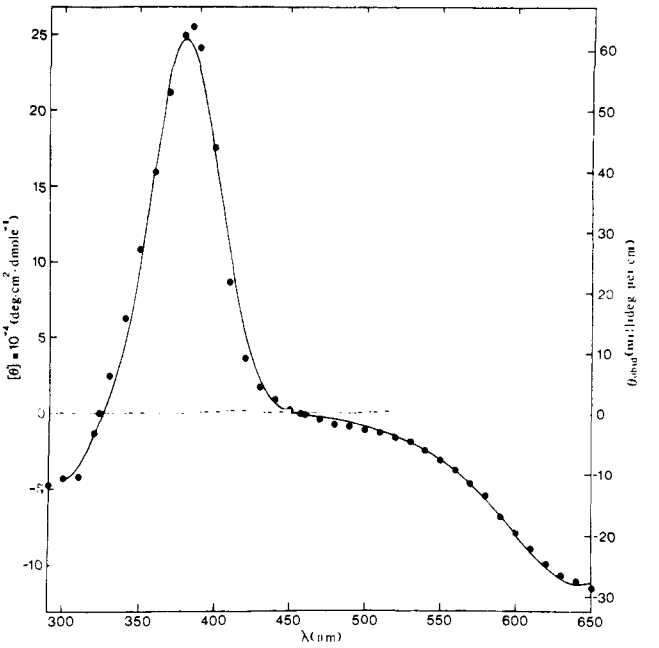

Figure 4. CD spectrum of the BV–HSA complex at pH 7.4. (Reproduced from ref 5, where details are given.)

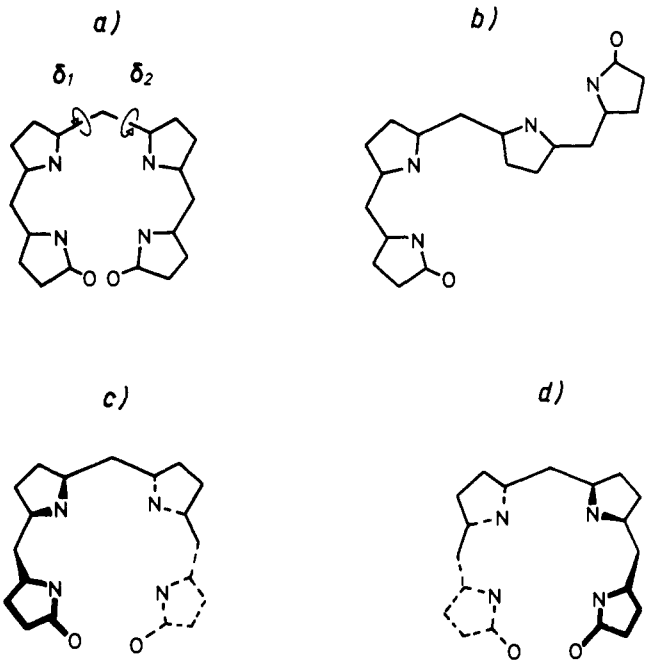

Figure 5. By considering the two dipyrromethene halves as rigidly planar, the conformations of bilirubin may be characterized by two dihedral angles δ_1 and δ_2, which independently may go from 0° to ±180°: (a) depicts the situation $\delta_1 \approx 0°$, $\delta_2 \approx 0°$; (b) corresponds to $\delta_1 = 0°$, $\delta_2 = \pm 180°$; (c) refers to $0° < \delta_1 < 90°$, $0° < \delta_2 < 90°$; (d) refers to $0° > \delta_1 > -90°$, $0° > \delta_2 > -90°$. In c the chirality is right-handed, in d left-handed. For equal absolute values of δ_1 and δ_2, c and d represent enantiomers.

Results for Bilirubin. We started out by performing calculations for left-handed bilirubin conformations δ_1/δ_2 = 0°/−10°, 0°/−30°, 0°/−60°, 0°/−90°, and 0°/−120°, focusing our attention on the sign and magnitude of the rotatory strengths and the energy splitting between the two longest wavelength transitions (see Figure 8 and Table III). It appears that the experimental energy splitting of about

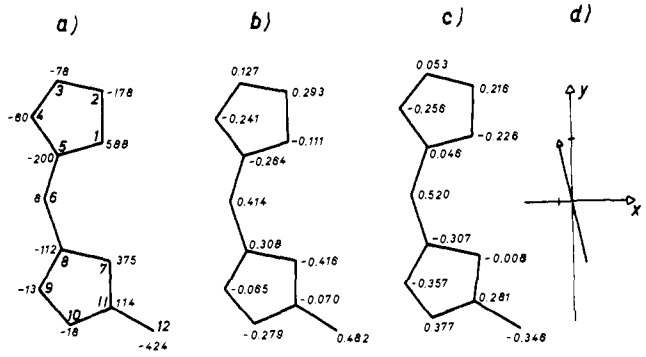

Figure 6. Computed data and numbering of atoms for dipyrromethene lactam. (a) The ground state charge distribution in units of $|e| \times 10^3$. (b) and (c) The LCAO coefficients of the highest filled and lowest unfilled MO's. The corresponding singly excited configuration makes a contribution of 0.87 to the first excited state. (d) The direction of polarization of the dipole velocity transition moment to the first excited state. Its absolute magnitude in au is 0.188.

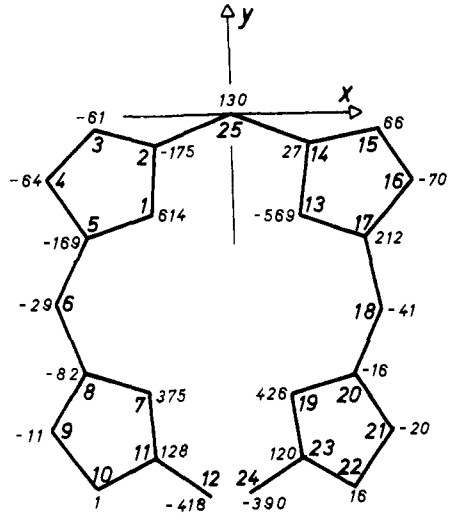

Figure 7. Adopted coordinate system and numbering of atoms in bilirubin and biliverdin. The dihedral angles δ_1 and δ_2 (Figure 5) correspond to a twist around bonds 2–25 and 25–14, respectively. In biliverdin carbon atom 25 takes part in the conjugation and nitrogen atom 13 is assumed to contribute only one (pseudo) π electron to the conjugated chromophore. Numbers indicate the ground state charge distribution in units of $|e| \times 10^3$ in biliverdin 30°/0° ($\delta_1 = 30°$, $\delta_2 = 0°$; $\beta_{2\text{-}25} = \beta_{25\text{-}14} \cos 30°$).

0.3 eV lies in the region between 0°/−30° and 0°/−60°. For $|\delta_2| > 60°$, it decreases rapidly. The computed absolute values of the rotatory strengths are of the correct order of magnitude. For the bilirubin of left-handed chirality, the predicted longest wavelength Cotton effect is always negative, followed by a positive one, as one would expect.[15-17] We conclude in a semiquantitative fashion that a conformation such as 0°/−50° is plausible for a left-handed conformer of bilirubin in the complex at pH 4. This agrees with a previous estimate on the basis of a simple dipole model.[3]

For $|\delta_2| < 30°$, the calculated CD spectrum becomes very unlike the experimental one (see Figures 2 and 8). This

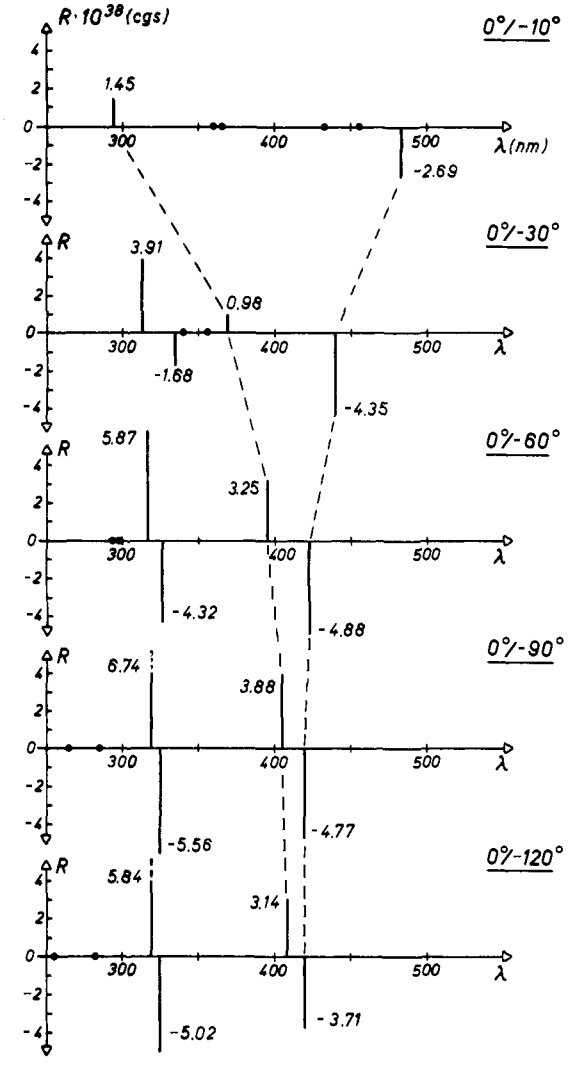

Figure 8. Calculated CD spectrum of bilirubin in the conformations δ_1/δ_2, as indicated. Rotatory strengths are in cgs units $\times 10^{38}$. The chirality is left-handed throughout. Notice in particular the decreasing exciton-type splitting of the two longest wavelength transitions, as δ_2 tends to −180°. The experimental situation (compare Figures 1 and 2) appears to lie between 0°/−30° and 0°/−60°. Conformations for which $\delta_2 > -30°$ ($|\delta_2| < 30°$) may be ruled out, also because of steric interference between the two carbonyl groups. On the other hand, in conformations for which $\delta_2 < -60°$ ($|\delta_2| > 60°$) the energy gap between the two first transitions becomes too small to agree with the experimental spectrum.

coincides with the fact that such conformations are energetically highly improbable, as the distances between the atoms of the carbonyl group of one dipyrromethene moiety and the carbonyl group of the other become smaller than the sum of the respective van der Waals radii (see Table IV). This repulsion is less obvious from the use of CPK molecular models.[3]

Since the energy splitting of the long-wavelength transitions decreases very markedly with the angle for $|\delta_2| > 60°$

Table I. Atomic PPP Parameters in Electron Volts[a]

Atom	p	I'_p	γ_p
N	1	−19.60	12.27
C	2	−9.00	10.53
C	11	−9.50	10.53
O	12	−14.00	14.50
N	13	−13.40*	12.27

[a] The value designated by an * refers to biliverdin only, in which the nitrogen atom 13 contributes only one $2p_\pi$ electron.

Table II. PPP Parameters for Bonds in Electron Volts[a]

Bond	p–q	β_{pq}	γ_{pq}	r_{pq}, Å
N–C	1–2	−2.50	7.65	1.370
C–C	2–3	−2.46	7.17	1.450
				1.520**
C–C	2–25	$-2.46 \lvert\cos \delta_1\rvert$*	7.23*	1.390*
C–O	11–12	−2.80	8.42	1.230
N–C	13–14	−2.50	7.65	1.370

[a] Values designated by an * refer to biliverdin only, by ** to bilirubin only.

Table III. Computed Quantities for the Two First Excited States in Bilirubin (BR) and the First Excited State in Biliverdin (BV)[a]

Molecule	Excited state	ΔE	$\langle\nabla\rangle_x$	$\langle\nabla\rangle_y$	$\langle\nabla\rangle_z$	$(r\times\nabla)_x$	$(r\times\nabla)_y$	$(r\times\nabla)_z$	f	$10^{38}R$
BR 0°/−30°	1	2.825	−0.09066	0.02200	0.07916	−0.3939	−0.5570	−2.7175		−4.35
	2	3.372	−0.00332	0.17078	−0.05072	0.1891	0.2674	−0.1334		0.99
BR 0°/−60°	1	2.931	−0.03626	0.06112	0.11369	−0.5226	−0.7391	−1.7312		−4.88
	2	3.135	−0.03015	0.17094	−0.11178	0.5067	0.7166	−0.4608		3.25
BR 0°/−90°	1	2.949	−0.00034	0.11310	0.11835	−0.5340	−0.7552	−1.1322		−4.77
	2	3.061	−0.06958	0.11674	−0.13520	0.6083	0.8603	−0.9392		3.88
BR 0°/−120°	1	2.948	0.02228	0.16342	0.08939	−0.4024	−0.5690	−0.7681		−3.71
	2	3.036	−0.11087	0.04657	−0.12580	0.5607	0.7929	−1.3843		3.15
BR 60°/−30°	1	2.737	−0.06149	−0.04491	−0.05741	0.2703	−1.6175	−2.0173		4.03
	2	3.304	0.01718	0.12522	−0.11517	0.3625	0.0181	0.3880		−0.70
BR −30°/−30°	1	2.949	−0.04748	0	0.1349	−0.6350	0	−1.9049	0.126	−4.93
	2	3.117	0	0.20081	0	0	0.8556	0	0.234	3.53
BV 30°/0°	1	1.760	−0.04467	0.03125	0.05542	−0.2457	0.4458	2.0487	0.062	5.04
BV −10°/−10°	1	1.579	−0.00466	0.01456	−0.03090	0.0575	−0.0033	2.5463	0.014	−3.21
BV 150°/0°	1	2.000	−0.14708	0.17131	0.03789	−0.1545	0.2967	0.3530	0.475	2.79
BV 150°/180°	1	2.054	0.26499	0.01496	−0.04645	0.1900	−0.3484	1.5985	0.641	−0.58

[a] The excitation energies ΔE are in eV, the components of the dipole velocity transition moments $\langle\nabla\rangle$ in au, and the rotatory strengths in cgs units. f values are indicated where they have been calculated.

Table IV. Distance between the Two Carbonyl Oxygen Atoms in Å for the Conformations of Bilirubin (BR) and Biliverdin (BV) Taken into Consideration[a]

	BR						
	0°/−10°	0°/−30°	0°/−60°	0°/−90°	0°/−120°	60°/−30°	−30°/−30°
r_{O-O}	2.13	3.38	5.72	7.86	9.53	2.83	6.05
	BV						
	30°/0°	−10°/−10°	150°/0°	150°/180°			
r_{O-O}	3.11	2.23	11.12	11.06			

[a] The van der Waals radius of oxygen is 1.40 Å.

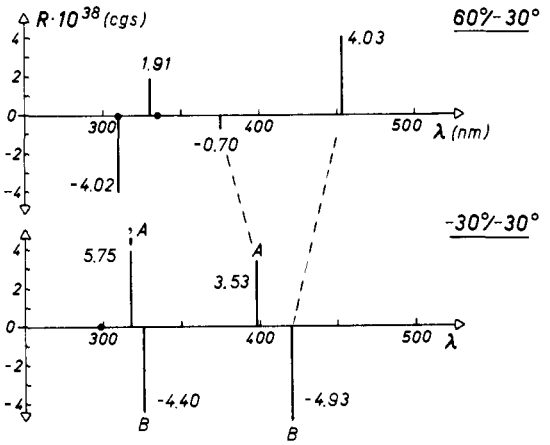

Figure 9. Calculated CD spectrum of bilirubin in the conformations 60°/−30° and −30°/−30°. The former case is comparable to 30°/0° (enantiomeric to 0°/−30°), the latter case to 0°/−60°. In the case −30°/−30° the molecule has exact C_2 symmetry, and the transitions may thus be precisely characterized by the irreducible representations A and B. A-type transitions are polarized parallel to the C_2 axis (along the y axis), B-type transitions perpendicularly (along the x, z axes). The general connection of the computed CD spectra of bilirubin to the "C_2-rule" is herewith established (ref 15–17).

(see above), the absolute values of the Cotton effects should also diminish,[3] due to mutual cancellation of ellipticities of opposite sign. The predicted CD spectra for the conformations −30°/−30° and 60°/−30° strongly resemble (Figure 9) those computed for 0°/−60° and 0°/30°, respectively (note the difference in chirality between the first and the second case). This allows the tentative general conclusion that for not too large absolute values of the angles the predicted spectrum for δ_1/δ_2 will be similar to $0°/(\delta_1+\delta_2)$, which, of course, is identical with $(\delta_1+\delta_2)/0°$. The sum of

Table V. Atomic Coordinates in Å for Bilirubin in the Case −30°/−30°

Atom p/q	x	y	z
1/13	∓1.322	−2.125	∓0.561
2/14	∓1.241	−0.878	0.000
3/15	∓2.524	−0.540	±0.587
4/16	∓3.340	−1.580	±0.368
5/17	∓2.574	−2.576	∓0.356
6/18	∓3.040	−3.813	∓0.784
7/19	∓0.995	−4.632	∓1.852
8/20	∓2.306	−4.767	∓1.479
9/21	∓2.793	−6.058	∓1.925
10/22	∓1.775	−6.666	∓2.550
11/23	∓0.623	−5.772	∓2.513
12/24	±0.494	−5.982	∓2.984
25	0.000	0.000	0.000

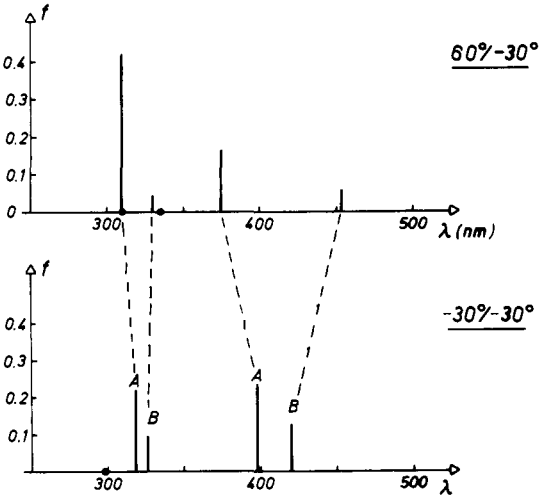

Figure 10. Calculated absorption spectrum for bilirubin in the conformations 60°/−30° and −30°/−30°. The f values are based on transition moments computed in the dipole velocity form.

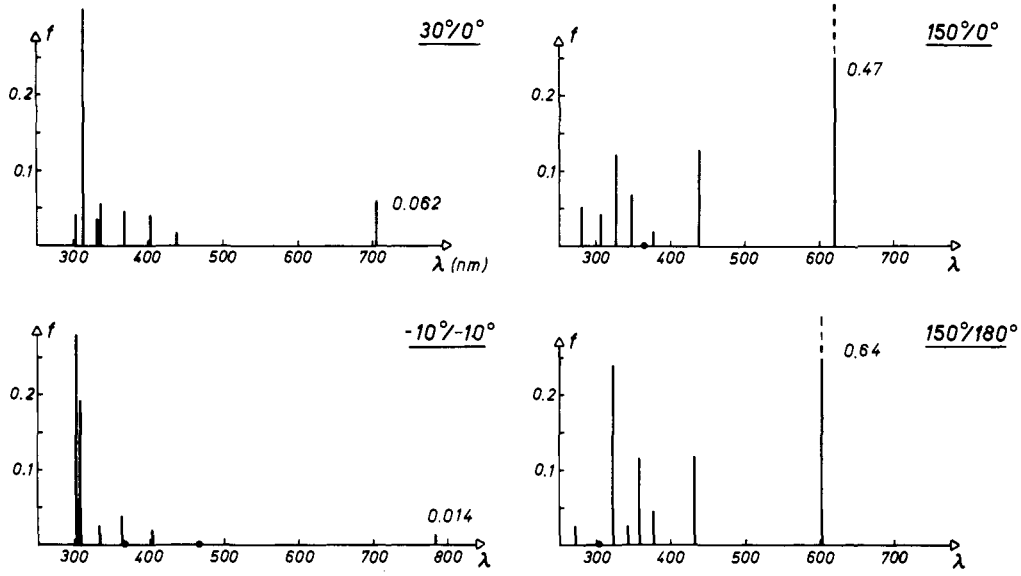

Figure 11. Calculated absorption spectrum of biliverdin in the conformations 30°/0°, −10°/−10°, 150°/0°, 150°/180°. The f values are based on transition moments computed in the dipole velocity form. Note the drastic increase in the oscillator strength of the longest wavelength transition in the "open chain," cases 150°/0° and 150°/180°.

the dihedral angles δ_1 and δ_2 thus becomes the characteristic parameter.

In conclusion, left-handed conformations, for which $(\delta_1 + \delta_2) \simeq -50°$ to $-60°$, seem to correspond to the low pH spectrum; right-handed conformations, for which $(\delta_1 + \delta_2) \simeq +30°$, correspond to the high pH spectrum. However, the absolute values of the observed Cotton effects at pH > 5 differ from the computed values to some extent.

Conformations for which $\delta_1 = \delta_2$ have exact C_2 symmetry (see Table V), and the transitions transform either as A or B. According to the "C_2-rule,"[15-17] the long-wavelength transitions of polarization A lead to positive Cotton effects if $(\delta_1 + \delta_2) < 0$ and those of polarization B to negative Cotton effects. For $(\delta_1 + \delta_2) > 0$, the situation is, of course, reversed. Calculated oscillator strengths for two different conformations are shown in Figure 10.

In the CD spectrum of the bilirubin–serum albumin complex there sometimes appears an additional weak Cotton effect around 500 nm, in front of the first strong long-wavelength transition.[3] In the CD spectrum of blood serum this band is stronger.[18] This band is not observed when charcoal-treated HSA is used. Such evidence suggests that it is not the bilirubin chromophore per se which is responsible for this Cotton effect, but possibly some type of interaction of the pigment with its environment. However, in order to test the possibility of a transition of the $n\pi^*$-type at long wavelengths in bilirubin, we have performed a CNDO calculation of the lower excited states of dipyrromethene. It turns out that the predicted longest wavelength $n\pi^*$ transition in dipyrromethene occurs at 320 nm, which indeed makes the appearance of an $n\pi^*$ transition in bilirubin beyond the long-wavelength $\pi\pi^*$ transitions quite improbable.

Results for Biliverdin. The observed red shift of the longest wavelength band is well reproduced by the calculation. The calculated absorption spectrum is here of particular interest (see Figure 11 and Table III). In the "closed" conformations −10°/−10° and 30°/0° the calculated oscillator strengths of the 300–400-nm band system are greater by an order of magnitude than the one for the long-wavelength 700-nm band. In the "open" conformations 150°/0° and 150°/180°, the situation is reversed, and the long-wavelength transition gains in intensity at the expense of the transitions at shorter wavelengths.

This situation may be rationalized in the following way.

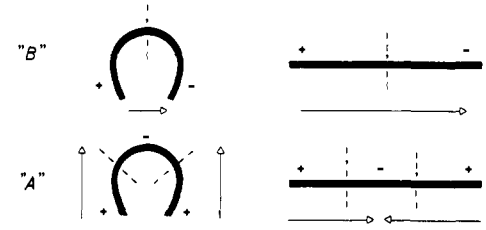

Figure 12. Partial and resulting transition moments for "A-type" and "B-type" transitions in "round-field" and "long-field" molecules.

The longest wavelength transition is a typical "B-type" transition with an odd number of nodes in the excited state. As shown in Figure 12, such a transition tends to have a relatively large electric dipole transition moment in the "open," or "long-chain" conformation. The computed strong transitions below 400 nm, on the other hand, which make the dominant contribution in the "closed," or "round-chain" conformations, must conceivably be of the "A-type" and have an even number of nodes in the excited state. Neither computed extreme seems to coincide with the experimental spectrum. From the measured relative intensities we could estimate an energetically unlikely conformation for biliverdin in the complex for which $|\delta_1 + \delta_2| \simeq 50$–$70°$. Alternatively, we cannot exclude the possibility of the presence of a mixture of "open" and "closed" forms in the biliverdin preparation used.

The computed CD spectrum of chiral biliverdin is less informative (Figure 13). The spectra of the extreme conformations (−10°/−10°, on the one hand, and 150°/180°, on the other) are hard to reconcile with measured data. These conformations may thus plausibly be ruled out. The absolute values of the results for both 30°/0° and 150°/0° show some degree of agreement with experiment (as far as the CD spectrum is recorded); in the latter case, the relative magnitude of the computed Cotton effects is possibly even better. From the sign of the experimental Cotton effects we conclude that biliverdin must be skewed in a left-handed conformation. Further computations are in progress in order to test the effect of different types of skewing of the chromophore on the theoretical data.

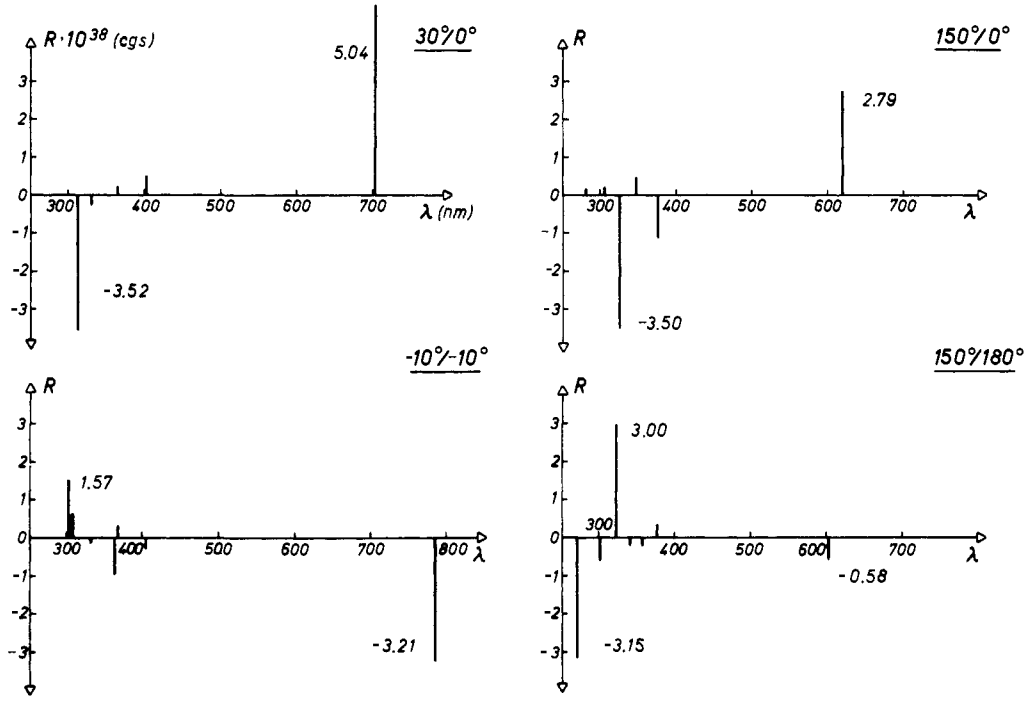

Figure 13. Calculated CD spectrum of biliverdin in the conformations 30°/0°, −10°/−10°, 150°/0°, 150°/180°. Rotatory strengths are in cgs units × 10^{38}. The wavelength scale is in nanometers. The conformations 30°/0° and 150°/0° have a chirality opposite to the one of the conformations −10°/−10° and 150°/180°. The sign of corresponding Cotton effects changes accordingly.

Conclusions

Both the absorption and CD spectra, but in particular the latter, of the complexes of bilirubin and biliverdin with human serum albumin indicate a highly specific mode of binding between the pigment and the protein. A change in pH (midpoint around pH 5) inverts the sign of these Cotton effects. One may assume that conformational changes in the protein invert the internal chirality of the bilirubin. We have attempted to characterize the relative change in geometry of these chromophores by just two dihedral angles, δ_1 and δ_2. The computed CD spectrum of bilirubin leads to the conclusion that right-handed conformations, for which $(\delta_1 + \delta_2) \simeq +30°$, are probable for the high pH form, while left-handed conformations, for which $(\delta_1 + \delta_2) \simeq -50°$ to $-60°$, correspond to the low pH form with charcoal-treated HSA. We are, of course, aware that our geometric characterization of the pigment may well be an oversimplification and that the individual dipyrromethene moieties may be skewed as well. Also, it is evident that the inversion process could not go through any intermediate conformations where steric interference becomes important. We nevertheless believe that these considerations give a semiquantitative indication of the geometry of the pigment in the complex, and, in particular, that the pigment is bound in a relatively tight helical conformation. In the case of the complex with biliverdin, conclusions are somewhat more difficult to arrive at, as indicated above.

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