Monatshefte für Chemie 121, 653 – 663 (1990)

Monatshefte für Chemie
Chemical Monthly

Reactivity of Pyrrole Pigments, Part 13 [1]: Identification of the Reaction Product Generated from Bile Pigments by the Superoxide Anion

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Summary. The UV/Vis spectra of the conjugated bases (NH deprotonation) of biliverdin IX α (*BV*), mesobiliverdin IX α (*MBV*), biliverdin IX α dimethyl ester (*BV – DME*) and mesobiliverdin IX α dimethyl ester (*MBV – DME*) are shown. They resemble those obtained for the reaction products of these biliverdins with superoxide anion ($O_2^{\cdot-}$). These results confirm that the bile pigments react with $O_2^{\cdot-}$ giving the lactam NH deprotonated conjugated bases and inducing $O_2^{\cdot-}$ dismutation. The spectrometric titrations of *BV*, *MBV* and their dimethyl esters show that the lactam NH of the vinyl substituted biliverdins is more acidic ($\Delta pK_a \cong 0.5$). The spectra of the lactam NH bisdeprotonated conjugated bases of the bilatrienes-*abc* studied (BV^{4-} and MBV^{4-}) can be obtained in *DMSO*/H_2O/OH^- systems of high basicity function ($H_- \cong 23$).

Because of the low oxidation potentials of BV^{3-} and of the corresponding trianion of bilirubin IX α (studied by voltammetry) an alternative metabolic degradative pathway is suggested for bilirubin, involving the interaction in lipophilic media with $O_2^{\cdot-}$ and oxidation of the conjugated base generated by NH deprotonation.

Keywords. Bilirubin metabolism; pK_a of biliverdins.

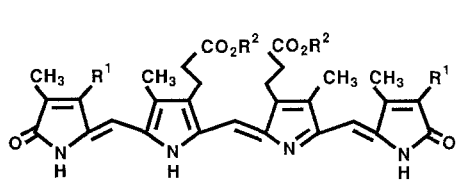
Reaktivität von Pyrrolpigmenten, 13. Mitt.: Identifizierung der Reaktionsprodukte von Gallenpigmenten mit Superoxydanion

Zusammenfassung. Es wurden die UV/Vis-Spektren der konjugierten Basen (NH-Deprotonierung) von Biliverdin IX α (*BV*), Mesobiliverdin IX α (*MBV*), Biliverdin-IX α-dimethylester (*BV – DME*) und Mesobiliverdin-IX α-dimethylester (*MBV – DME*) bestimmt. Sie ähneln denen der Reaktionsprodukte, die man bei der Umsetzung dieser Biliverdinverbindungen mit Superoxydanion ($O_2^{\cdot-}$) erhält. Damit wird bewiesen, daß die Reaktion über eine N-Deprotonierung der Lactamgruppe verläuft, an die sich eine $O_2^{\cdot-}$-Dismutation anschließt. Die spektrometrische Titration von *BV*, *MBV* und ihrer Dimethylester zeigt, daß der Lactamstickstoff in den vinylsubstituierten Biliverdinderivaten eine höhere Azidität aufweist ($\Delta pK_a \cong 0.5$). Die Spektren der bis-deprotonierten konjugierten Basen der Bilatriene-*abc* (BV^{4-} und MBV^{4-}) wurden in *DMSO*/H_2O/OH^--Systemen ($H_- \cong 23$) erhalten.

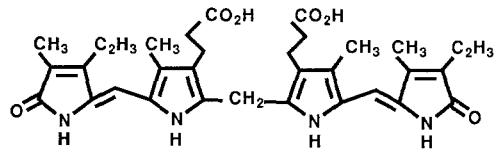
Unter Berücksichtigung der geringen Redoxpotentiale von BV^{3-} und des entsprechenden Trianions von Bilirubin IX α (voltammetrische Bestimmung) wird ein neuer metabolischer Abbau für Bilirubin IX α vorgeschlagen: primäre Deprotonierung in lipophilem Medium mit $O_2^{\cdot-}$ und nachfolgender Oxydation.

Introduction

We have recently [2] established by cyclic voltammetry the nature of the chemical interaction between the superoxide anion ($O_2^{\cdot-}$) and some bile pigments: Clearly,



	R^1	R^2
MBV	C_2H_5	H
BV	C_2H_3	H
MBV-DME	C_2H_5	CH_3
BV-DME	C_2H_3	CH_3



BR

the lactam hydrogen of the bilatrienes-*abc* and biladienes-*ac* is sufficiently acidic to induce superoxide dismutation, according to the well known overall equation [3]

$$2\,O_2^{\cdot-} + 2\,HA \rightarrow 2\,A^- + H_2O_2 + O_2.$$

Our interpretation contradicts the one given by Manitto et al. to their experimental results obtained by interaction of $O_2^{\cdot-}$ with biliverdin IX α (*BV*) and its dimethylester (*BV*−*DME*) [4]. Manitto's interpretation was based on the formation of a stable radical anion according to the following scheme:

$$O_2^{\cdot-} + BV^{2-}\ (\text{or } BV-DME) \leftrightarrows [BV---O_2]^{3\cdot-}\ (\text{or } [BV-DME---O_2]^{\cdot-})$$
$$\downarrow\uparrow$$
$$BV^{3\cdot-}(\text{or } BV-DME^{\cdot-}) + O_2.$$

The UV/Vis spectra of the supposed radical anions had been previously reported [4]. The results presented here show that actually these spectra correspond to the anions obtained by deprotonation at the lactam hydrogen of bilatrienes-*abc*.

Results and Discussion

Identification of the Supposed [4] *Radical Anions* $BV^{3\cdot-}$ *and* $BV-DME^{\cdot-}$ *as the Corresponding* NH *Deprotonated Anions* (BV^{3-} *and* $BV-DME^-$)

The UV/Vis spectrum of *BV*−*DME* in dimethylformamide (*DMF*) with an excess of 1,1,3,3-tetramethylguanidine (*TMG*) (see Fig. 1) is the same as that obtained by solubilisation of *BV*−*DME* in a $O_2^{\cdot-}$ solution in *DMF* [obtained by electrolysis at controlled potential: −0.8 V (s.c.e.)]. The small differences between this spectrum and the one reported for the supposed $BV-DME^{\cdot-}$ [4a], obtained in *DMSO*/KO_2, are due to solvent effects, as shown by the spectrum attributed to *BV*−*DME* in *DMSO* containing an excess of *TMG* (see spectrum 10 in Fig. 2). Similarly, the reported UV/Vis spectrum of *BV* in *DMSO*/KO_2 [4a], attributed to $BV^{3\cdot-}$, is the

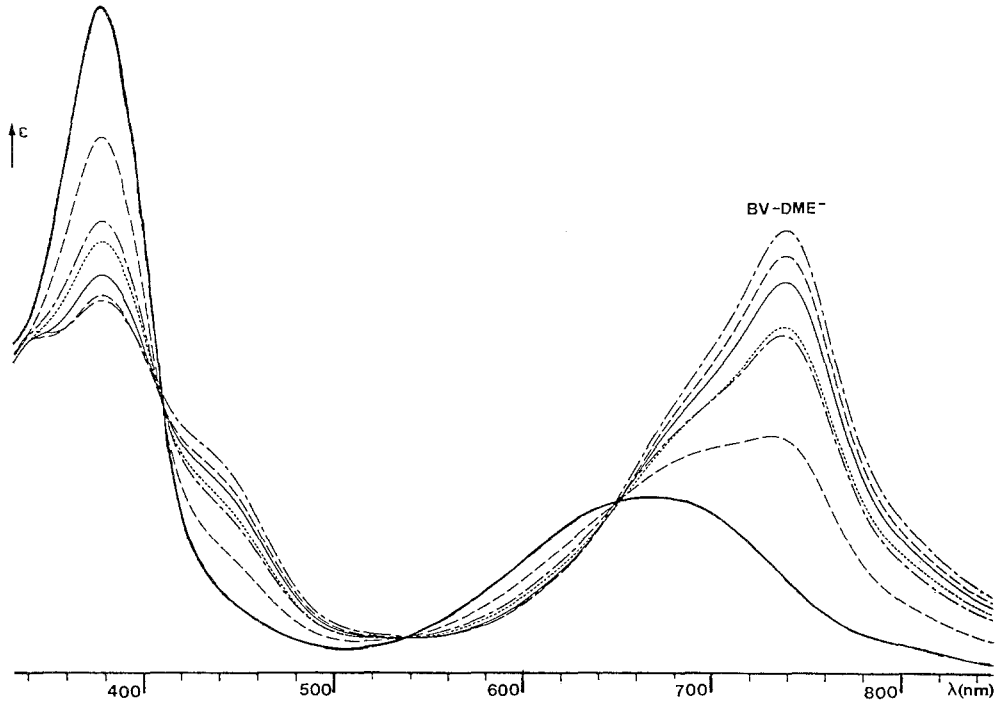


Fig. 1. UV/Vis spectra of *BV−DME* $1.6 \cdot 10^{-5}$ mol l^{-1} solutions in *DMF* at several concentrations of tetramethylguanidine (between 0 and 0.2 mol l^{-1})

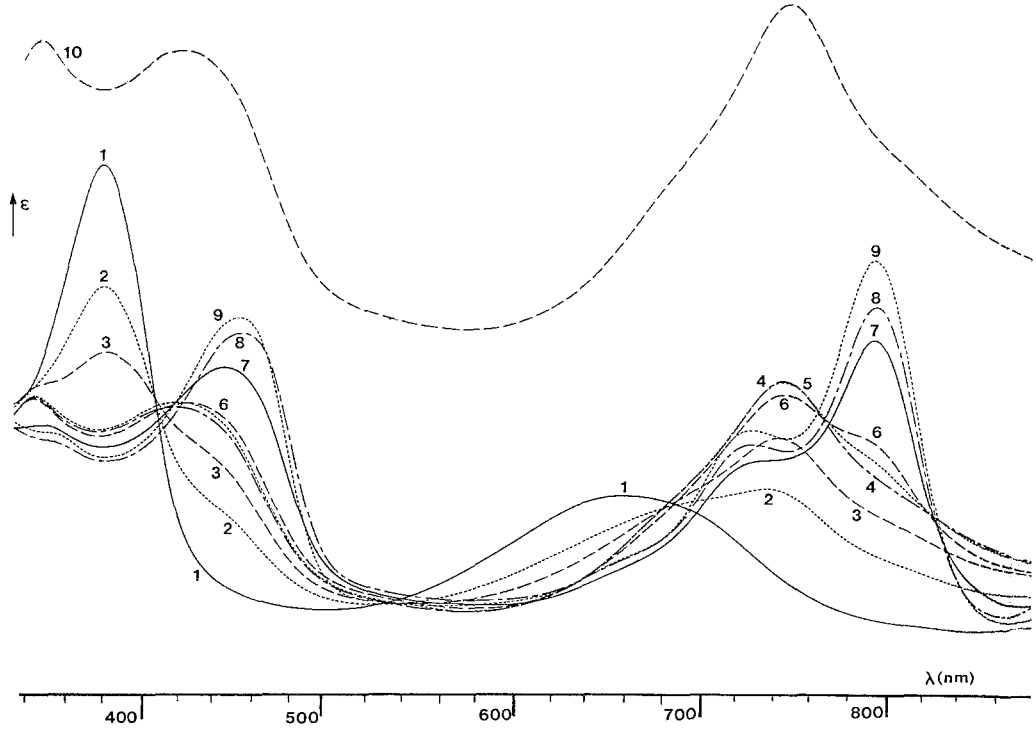


Fig. 2. UV/Vis spectra of *BV−DME* $1.6 \cdot 10^{-5}$ mol l^{-1} in *DMSO* alkaline solutions. 1–9: $Bu_4N^+ OH^-$, 0, $3 \cdot 10^{-5}$, $6.6 \cdot 10^{-5}$, $1.8 \cdot 10^{-4}$, $2.4 \cdot 10^{-4}$, $3.0 \cdot 10^{-4}$, $4.2 \cdot 10^{-4}$, $6.6 \cdot 10^{-4}$, and 0.08 mol l^{-1}, respectively. 10: *TMG* 0.16 mol l^{-1}

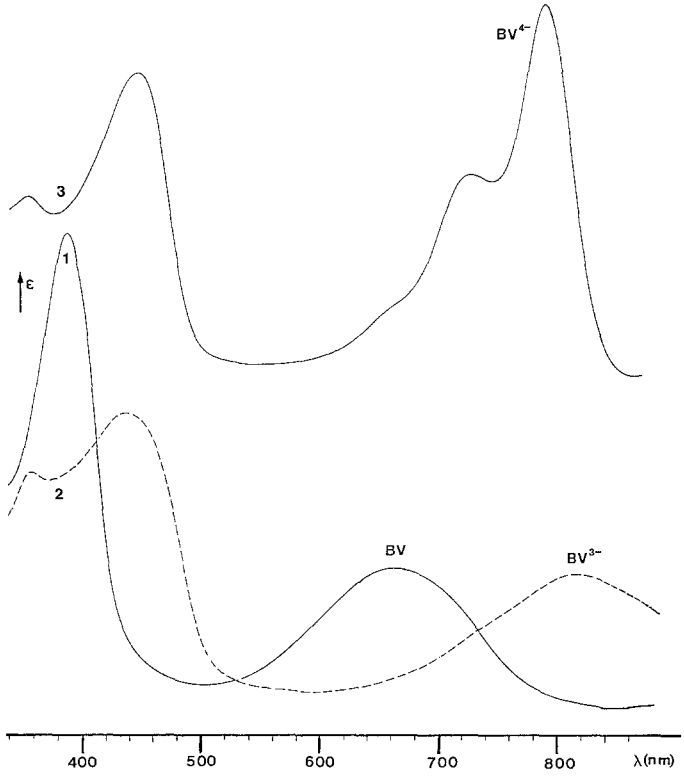


Fig. 3. UV/Vis spectra of *BV* solutions in *DMSO*. 1: *BV* $1.84 \cdot 10^{-5}$ mol l^{-1}. 2: *BV* $1.84 \cdot 10^{-5}$ mol l^{-1} and *TMG* 0.2 mol l^{-1}. 3: *BV* $1.65 \cdot 10^{-5}$ mol l^{-1} and Bu_4N^+ OH^- $6.6 \cdot 10^{-3}$ mol l^{-1} (*DMSO* 99 mol%)

same as that obtained in *DMSO* with a *TMG* excess (see spectrum 2 in Fig. 3). In conclusion, the first products formed by reaction of these biliverdins with $O_2^{\cdot-}$ are the conjugated bases formed by CO_2H and lactam NH deprotonation, and not the radical anions.

The similarity of the spectrum of *BV* in *DMF*/KO_2 with that of the transient obtained by pulse radiolysis of BV^{2-} solutions, attributed to $BV^{3\cdot-}$ [5], was already noted by the authors of Ref. [4]. In our opinion, the identification of this radical anion obtained by pulse radiolysis requires additional evidence owing to the increase of "transient" basicity originated during the pulse radiolysis and the relatively low pK_a values of *BV* (towards BV^{3-}) in water solutions (see below).

In order to clarify whether lactam NH mono or bisdeprotonation occurs by interaction with $O_2^{\cdot-}$, a more detailed study of the deprotonation processes of biliverdins was undertaken (see the summary of results in Table 1).

pK_a Values of BV and MBV and Effect of the Vinyl Substitution on the NH *Acidity*

The UV/Vis spectra of mesobiliverdin IX α (*MBV*) in all tested solvents, both with or without a base is always similar to those of biliverdin IX α (*BV*) under the same conditions; the same is true for their dimethyl esters (*MBV* − *DME* and *BV* − *DME*). However, as it is already known, the vinyl substitution results in bathochromic shifts of the two bands (see Table 1).

Table 1. Spectroscopic data of biliverdins in several solvent systems and in the presence of alkali excess

Substance	Solvent	λ_{BH} (nm)	ε_{BH}	Base (mol l^{-1})	λ_{B-} (nm)	ε_{B-}	Type of B^-
MBV	H_2O[a]	363	48 000	NaOH (2.0)	367	43 100	MBV^{3-}
		657	12 100		746	11 800	
	DMF	371	50 100	*TMG* (0.8)	≅419		MBV^{3-}
		628	14 100		≅770		
	DMSO	373	51 900	*TMG* (0.8)	≅414		MBV^{3-}
		632	15 600		≅760		
	DMSO/H_2O	368	46 900	$Me_4N^+ OH^-$ (0.022)	375	34 400	MBV^{3-}
	(30 mol%)	650	14 400		695 sh	10 700	
					768	18 800	
	DMSO/H_2O	372	49 900	$But_4N^+ OH^-$ (0.012)	419	37 000	MBV^{4-}
	(98.1 mol%)	632	15 100		631 sh	8 000	
					689	23 900	
					750	46 200	
BV	H_2O[a]	375	45 100	NaOH (2.0)	384	42 800	BV^{3-}
		674	12 200		761	13 200	
				$Me_4N^+ OH^-$ (2.0)	390	38 100	BV^{3-}
					725 sh	11 300	
					797	18 100	
	DMF	381	49 000	*TMG* (0.4)	≅443		BV^{3-}
		659	13 600		≅809		
	DMSO	383	52 300	*TMG* (0.2)	432	32 500	BV^{3-}
		662	15 400		813	14 800	
	DMSO/H_2O	382	46 500	$Me_4N^+ OH^-$ ($1.9 \cdot 10^{-3}$)	394	33 400	BV^{3-}
	(30 mol%)	683	13 400		730 sh	9 000	
					800	13 600	
	DMSO/H_2O	383	47 000	$Bu_4N^+ OH^-$ ($6.6 \cdot 10^{-3}$)	460	30 000	BV^{4-}
	(99.0 mol%)	660	13 400		650 sh	8 200	
					736	18 800	
					802	31 900	
MBV−DME	*DMF*	369	52 100	*TMG* (0.03)	324 sh	29 500	*MBV−DME*$^-$
		667	14 100		372	27 100	
					418 sh	12 500	
					646 sh	24 500	
					703	49 400	
	DMSO	371	52 400	*TMG* (0.14)	325	30 100	*MBV−DME*$^-$
		636	15 400		386	24 700	
					644 sh	19 600	
					707	43 800	
BV−DME	*DMF*	379	53 200	*TMG* ($3 \cdot 10^{-3}$)	342 sh	26 500	*BV−DME*$^-$
		667	14 100		379	29 800	
					448 sh	15 900	
					676 sh	20 700	
					740	35 500	
	DMSO	382	50 100	*TMG* (0.1)	345	25 500	*BV−DME*$^-$
		662	15 600		422	24 800	
					668 sh	11 600	
					746	29 200	

[a] 2 Base equivalents were added to the initial bilatriene-*abc* solution

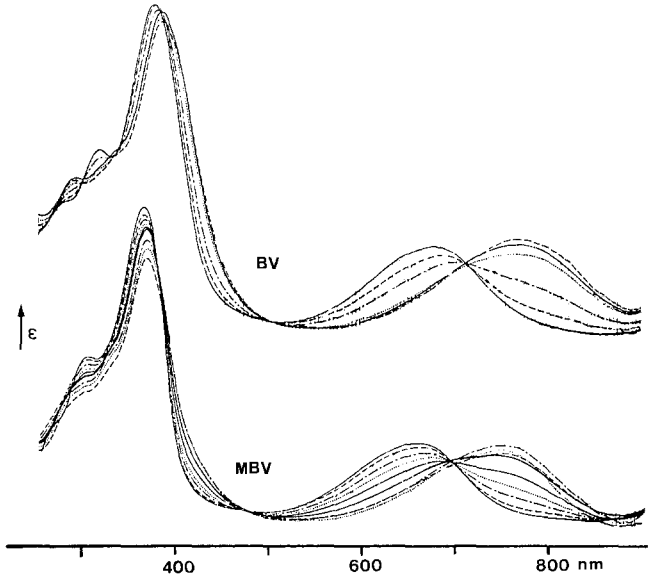


Fig. 4. UV/Vis spectrometric titration in H_2O/NaOH (NaOH concentrations between two equivalents and 2 mol l^{-1}) of biliverdins with propionic acid substituents at C8 and C12. *MBV*, $1.48 \cdot 10^{-5}$ mol l^{-1}. *BV*, $1.96 \cdot 10^{-5}$ mol l^{-1}

The spectrum of BV^{3-} in H_2O/NaOH has already been described in the literature [8]. However, its pK_a had not been measured. Using the basicity function (H_-) values reported in the literature [6a] for the system H_2O/NaOH, we have measured pK_a values of 12.7 and 13.1 for *BV* and *MBV* respectively (see Fig. 4). In the system *DMF/TMG* absolute pK_a values cannot be measured because the corresponding activity values are not known; however, a difference of 0.7 in the corresponding log[Base] at $\log[AH]/[A^-] = 0$ was also shown between *BV*−*DME* and *MBV*−*DME*. For *BV* and *MBV*, the *DMF/TMG* system is not basic enough to obtain complete lactam NH monodeprotonation; nevertheless, the system can be shifted to a larger extension towards the conjugated base in the case of *BV* than for *MBV*. A similar behaviour is also shown in the *DMSO/TMG* system. In this case, owing to the differences between the solvents *DMSO* and *DMF* [7], *BV* can be completely shifted to its conjugated base, BV^{3-}, whereas *MBV* is only partially deprotonated. These results show that the vinyl containing biliverdin is more acidic than its ethyl-containing counterpart.

The pK_a values reported here for *BV* and *MBV* are lower than expected if the pK_a values of other bilatrienes-*abc* without propionic acid substituents were taken into account [9]: e.g. 3,8,12,17-tetraethyl-2,7,13,18-tetramethyl-bilin-1,19-dione has a $pK_a = 14.7$, in the system *DMSO*/H_2O/$(CH_3)_4N^+$ OH^-. Our spectrometric titrations of *BV* and *MBV* in the same solvent system (30 mol % *DMSO*) yield also similar pK_a values (13.9 and 14.6 respectively), i.e. the measured pK_a values seems to be solvent dependent, probably because of the non-linear relationship to the acidity of the indicators of the H_- scale.

Our results show that, in water, the pK_a values for deprotonation of *BV* and *MBV* to their trianions are lower than expected [9]. Extrapolation of this behaviour to the 2,3-dihydrobilatrienes-*abc*, suggests that pK_a values in the order of 10 should be expected when they contain propionic acid substituents at C8 and C12. A pK_a value close to 10 is of significance in relation to the phytochrome problem.

Which of the two lactam NH's of *BV* (the one belonging to ring A, with the *endo*-vinyl or the one in ring B, with an *exo*-vinyl) is deprotonated first has not been established, although we are currently comparing the symmetrical isomers III α and XIII α. These results could be important in relation with the effectiveness of the hydrogen bonds in which free lactam NH's participate, both in biliverdins and bilirubins. For the last, on the basis of their ^{1}H-NMR chemical shifts, a higher capacity to hydrogen-bond (and therefore dimerization in the adequate solvents) has been recognized for the NH in the *endo*-vinyl-containing lactam using [11].

The Deprotonation of Two NH *Groups and the Pattern of the Spectra of the* NH *Mono- and Bisdeprotonated Bases*

Unlike the former experiments, in the system *DMSO*/H_2O/R_4N^+ OH^- (with $R = CH_3$ or n-C_4H_9) in solutions with low H_2O content (< 5 mol%), i.e. with high H_- values, no isosbestic points could be obtained (see example of spectra 1–9 in Fig. 2): At very low OH^- concentration (spectra 1–5) the spectra show the same pattern as with an excess of *TMG* in *DMSO* or *DMF* (Fig. 1 and spectrum 10 in Fig. 2); i.e., the NH monodeprotonated conjugated base is generated. However, as the OH^- concentration increases, new bands appear at different wavelengths and with different oscillator strengths. The transition from the bands corresponding to NH monodeprotonation to the new bands appears at very high H_- values (> 20): We attribute these bands to the conjugated base obtained by deprotonation of the two lactam NH's. At such high H_- values, using OH^-, dimethyl ester saponification occurs very quickly. In fact, at these conditions, the spectra of *BV*−*DME* and *MBV*−*DME* are practically the same than the ones obtained from *BV* and *MBV* respectively (compare the spectrum 9 of Fig. 2 and the spectra BV^{4-} of Fig. 3).

To our knowledge, the bisdeprotonated (NH) conjugated base of bilatrienes-*abc* has not previously been described: The dianion reported in the literature [10 a] was later identified by the same authors as a monodeprotonated metal complex [10 b]. However, our results do not contradict those reported in the literature because no experiments at $H_- > 20$ have been reported [9].

Important bathochromic shifts are produced by solvent effect: e.g. the spectra of BV^{3-}, and MBV^{3-} show the same pattern in H_2O, *DMF* and *DMSO*, but compared to their acid forms the bathochromic shifts are much more important in the aprotic solvents: This effect is higher for the vinyl substituted *BV* (in *DMSO* about 150 nm and 50 nm for the low and high energy bands respectively: See Table 1 and compare Figs. 3 and 4).

A counter-anion effect is also observed. The BV^{3-} spectra in the case of tetraalkylammonium cations show a shoulder at about 700 nm both in *DMSO* and H_2O, which does not appear with other counter-ions such as H_2O/NaOH or *DMSO*/*TMG*. This effect of the counter-cation on the UV/Vis spectra is probably the result of an indirect influence on the biliverdin structure: e.g., an effect on the conformational equilibria, which could also explain such pK_a differences.

The dramatic differences in the spectra of BV^{4-} and MBV^{4-} as compared to those of BV^{3-}, MBV^{3-}, BV^{2-}, MBV^{2-}, *BV*, *MBV*, *BV*−*DME* and *MBV*−*DME*, suggest an important configurational and conformational change for the bilatriene-

abc structure; i.e., a "stretched" arrangement for the NH bisdeprotonated products and a "helical" one for the rest [12]. However, the uniqueness of the spectra of $BV-DME^-$ and $MBV-DME^-$, and of those of the conjugated bases of "non-polar", alkyl-substituted bilatrienes-*abc* [9], for which one should also expect a helical structure on the basis of chemical reasoning, invalidates any simple explanation about the structure of the conjugated bases of biliverdins.

The present results suggest that, in terms of their band pattern, three types of spectra can be obtained for the conjugated bases of bilatrienes-*abc*. As it is already known, bilatrienes-*abc* with propionic acid substituents at C8 and C12, their dicarboxylic salts and their dimethyl esters show very similar UV/Vis spectra [8]. We have shown here how the UV/Vis spectra of the trianion of bilatrienes-*abc* with two propionic acid substituents at C8 and C12 have this same pattern, but with a strong bathochromic shift of the low energy band (see Table 1 and Figs. 3 and 4). A second "type" of spectra is that of the conjugated bases obtained from deprotonation of the two lactam NH groups (BV^{4-} and MBV^{4-}: see Table 1 and compare the corresponding spectra of Figs. 2 and 3): A similar pattern is obtained for all NH bisdeprotonated conjugated bases, independently of whether they are dianions or tetraanions. The spectra of the monoanions of the dimethyl esters seem to belong to a third "type"; i.e., the conjugated base obtained by NH monodeprotonation is very different for *BV* or *MBV* compared to $BV-DME$ *or* $MBV-DME$ (see Table 1 and compare the corresponding spectra of the figures). The spectra reported in the literature [9] for the conjugated bases (monoanions) of other bilatrienes-*abc* (biliverdins and 2,3-dihydrobiliverdins) without propionic acid groups at C8 and C12 belong also to this third "type".

Deprotonation of Bilirubins (Biladienes-ac)

A spectrometric titration of biladienes-*ac*, unlike their partial models the dipyrrin-1(10*H*)-ones [13], is very difficult. The spectra of neutral bilirubins are much more solvent dependent themselves, on the other hand, for bilirubins, deprotonation of NH lactam groups is not reversible or only partially reversible. This irreversibility is due to the autoxidation processes which decompose bilirubin at high *pH* values even in the presence of very small amounts of oxygen [8]. Furthermore, it is difficult to establish whether the changes in the spectra (originated from conformational or configurational changes) are the result of the formation of the carboxylate salts, or of NH deprotonation. In addition, at high basicity values, other chemical processes can occur, e.g. in the system $DMSO/H_2O/OH^-$ at H_- values above 20 we could observe a partial isomerization to biladienes-*ab* (biliviolins) as well as scrambling to symmetrical isomers.

The spectroscopic and electroanalytical results show that the interaction between biliverdins, bilirubins and one dipyrrin-1(10*H*)-one [2] with $O_2^{\cdot-}$ is the same for the three types of linear polypyrroles. Consequently, taken into account the pK_a values of the dipyrrin-1(10*H*)-ones [13] and the identification of the lactam NH monodeprotonated products of biliverdins reported here, we can estimate that the bile pigment proton induced dismutation of $O_2^{\cdot-}$ produced by the lactam hydrogen occurs if it shows a pK_a below 17–18. This value is of the same order of magnitude as that of other substances which generate proton induced dismutation of $O_2^{\cdot-}$ [3].

*Oxidation of the Conjugated Bases of Bile Pigments (*NH *Deprotonated) and Relevance of these Results to Some Biological Aspects of Bilirubins and Biliverdins*

In the case of the lipophilic bilirubin (*BR*) we have speculated about its possible biological role as $O_2^{\cdot-}$ scavenger [2]. On the other side the results of Manitto [4b] suggest a relationship between bile pigments and oxidative electron transfer processes. Simple chemical reasoning shows that the anions obtained by NH deprotonation of bilirubins and biliverdins must be easier to oxidize: In fact, chemical evidence has been reported in this respect for bilirubin [8].

In a voltammetry study reported in the literature [14], it was shown that *BR* is much easier to oxidize in the presence of a *TMG* excess, which was interpreted as due to a lower potential for the dicarboxylate salts. In order to clarify the effect of bases on the oxidative behaviour of bile pigments, we have performed a simple voltammetric study on the effect of pyridine or *TMG* addition upon the anodic oxidation (Pt) of *BR*, *BR−DME* and *BV−DME* in *DMF*. Our results indicate that the addition of pyridine produces changes in the voltammogram which can be attributed to the generation of different associated or conformational forms in solution: e.g. the change in the peak potential values of *BR−DME* is of the same order of magnitude as in the case of *BR*, which, in the presence of a pyridine excess, must exist in the form of BR^{2-} (see Fig. 5). The addition of a *TMG* excess of *DMF* solutions of *BR* and *MBV−DME* produces a dramatic shift to the oxidation potential to lower potential values (easier to oxidize) in both cases (see Fig. 5). In conclusion, the conjugated base of bile pigments generated by NH deprotonation shows a much lower oxidation potential. This low oxidation potential explains on

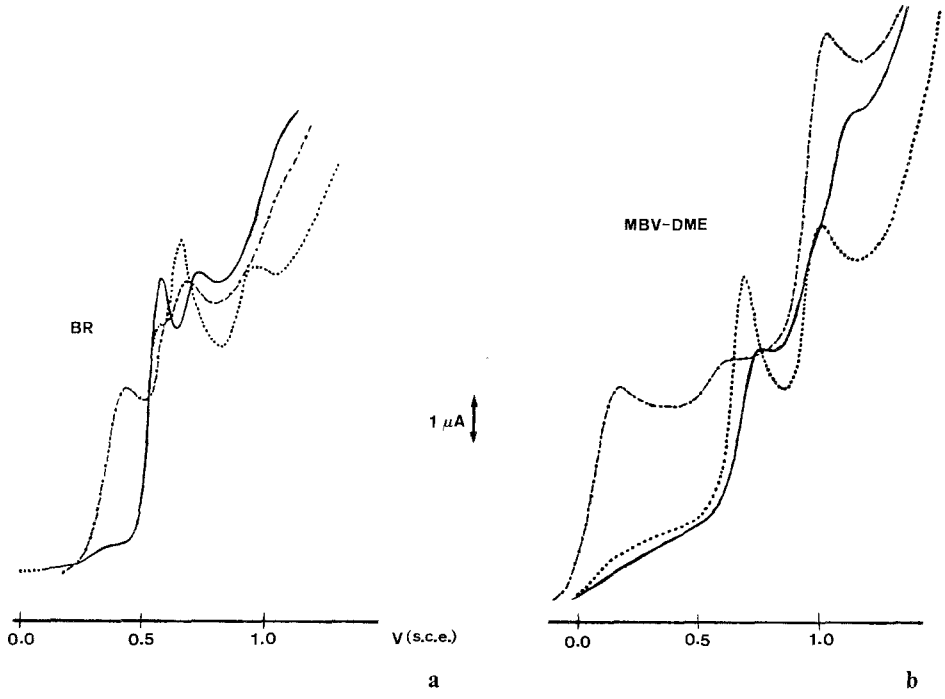


Fig. 5. Voltammograms in *DMF* of (**a**) bilirubin IXα dimethyl ester and (**b**) *MBV−DME*. ——— $5 \cdot 10^{-4}$ mol l^{-1} *DMF* solutions, 0.1 mol l^{-1} $LiClO_4$. ····· after addition of pyridine ($\cong 1 \cdot 10^{-3}$ mol l^{-1}). −·−·−·− after addition of *TMG* ($\cong 1 \cdot 10^{-3}$ mol l^{-1})

the one side the effect reported in Ref. [4 b] about the cytochrome *c* reduction by the product obtained by the interaction of $O_2^{\cdot-}$ and *BV*; i.e., BV^{3-} according to our results; on the other side, it suggests for bilirubin an alternative degradative pathway to that of the biliary excretion and affords a possible explanation for the elusive enzymatic system, which oxidizes bilirubin to polar, water-soluble products [8, 15]. Bilirubin in a lipophilic medium would react with $O_2^{\cdot-}$ to give its NH deprotonated conjugated base, which would be easily oxidized giving polar, more water-soluble products. In this sense it must be pointed out that an increase in the cytochrome P-450 levels reduces the plasma bilirubin levels in jaundiced Gunn rats, which had suggested a role of cytochrome P-450 in facilitating the elimination of bilirubin from body in the absence of glucuronidation [16].

Acknowledgement

This work is part of the CAICYT research program 459-84.

Experimental

The preparation and properties of biliverdin IX α (*BV*), mesobiliverdin IX α (*MBV*), and their dimethyl esters (*BV−DME* and *MBV−DME*) are described in the literature [8, 17, 18].

The UV/Vis spectra were recorded on a Perkin-Elmer Lambda 5 instrument. The pK_a values were determined from the spectrometric titration in basic solvent systems (see text), whose H_- values are described in the literature [6]. For more experimental details on this pK_a determination see Ref. [19].

Voltammetric anodic curves were obtained in the absence of O_2 from $5 \cdot 10^{-4}$ mol l^{-1} substrate solutions in *DMF* containing 0.1 mol l^{-1} $LiClO_4$, using a Pt ball of 4.4 mm^2, a saturated calomel-mercury electrode as reference and a Pt sheet as cathode.

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Received January 9, 1990. Accepted February 1, 1990