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Studies of the conformation of bilirubin and its dimethyl ester in dimethyl sulphoxide solutions by nuclear magnetic resonance

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The conformation of bilirubin and its dimethyl ester in dimethyl sulphoxide (DMSO) was investigated by n.m.r. spectroscopy. The chemical shifts of the pyrrole NH and Lactam protons of bilirubin and its dimethyl ester in DMSO indicate a strong interaction with the solvent. Inter-proton distances were calculated from nuclear Overhauser effects (NOE), selective and non-selective relaxation times (T_1) and rotational correlation times taken from ^{13}C relaxation times. The interproton distances indicate that the conformation of the skeleton of bilirubin and its dimethyl ester in DMSO is similar to that of bilirubin and mesobilirubin in the crystalline state and in chloroform solutions, except for a possible slight twist of the pyrrolenone rings about the methine bonds, which may be a consequence of solvation of the NH groups by DMSO. Unlike in chloroform solutions, no direct hydrogen-bonding occurs between the carboxylic acid and the lactam groups of bilirubin in DMSO, as shown by the absence of an NOE between these groups. The fast exchange of the pyrrole NH protons with ^2H shows that no hydrogen-bonding occurs between these protons and the propionic residues, in line with their solvation by DMSO. From the above results, and from the slowness of the internal motion of the propionic residues of bilirubin and its dimethyl ester, it is concluded that these residues are tied to the skeleton via bound solvent molecules.

The role of bilirubin in neonatal jaundice leading to kernicterus, and the need to elucidate the mechanism of phototherapy, have stimulated investigations of the structure of this compound in solution (see, e.g., Brodersen, 1980, and references therein). Recent measurements of T_1, NOE and exchange rates of the labile protons of bilirubin and some of its derivatives have aided the establishment of the conformations of these compounds in chloroform solutions (Kaplan & Navon, 1981*a*). In these studies, the structures of mesobilirubin and bilirubin in chloroform solutions were shown to involve a system of internal hydrogen-bonding that is similar to that found in the crystalline state (Becker & Sheldrick, 1978; Bonnett *et al.*, 1978; Le Bas *et al.*, 1980). Estimation of interproton distances from the n.m.r. relaxation data (Kaplan & Navon, 1981*a*) gave results that deviated significantly from values derived from earlier X-ray data (Bonnett *et al.*, 1978), but agreed well with more recent X-ray results (Le Bas *et al.*, 1980). Bilirubin dimethyl ester has been shown to be dimeric in chloroform solutions over a large concentration range (Holzwarth *et al.*, 1980; Kaplan & Navon, 1981*a*). Little is known about the conformation of bile pigments in polar solvents. Recent ^{13}C relaxation studies of bilirubin and its derivatives in DMSO solutions have shown that the motional freedom of the propionic residues of bilirubin and its dimethyl ester is very limited compared with its value in model compounds, and that no self-aggregation of these pigments occurs in this solvent (Kaplan & Navon, 1981*b*).

In the present work the conformations of bilirubin and its dimethyl ester in DMSO were studied by using their selective and non-selective proton n.m.r. spin-lattice relaxation times, NOE and rates of exchange (see Fig. 1 for the structures of the compounds).

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Abbreviations used: T_1, spin-lattice relaxation time; T_1^{ns}, non-selective T_1; T_1^s, selective T_1; NOE, nuclear Overhauser effect; DMSO, dimethyl sulphoxide; VBA, vinylneoxanthobilirubinic acid.

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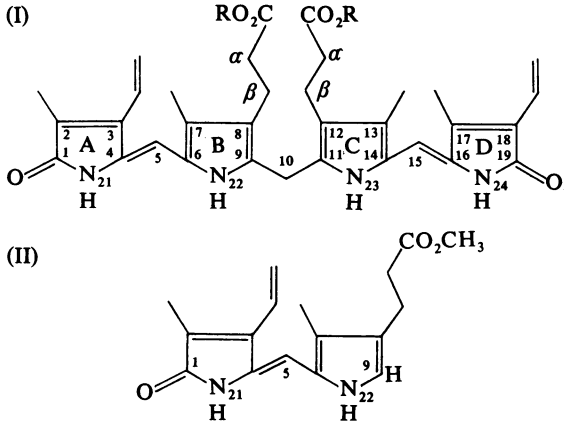

Fig. 1. *Schematic structures of bilirubin (I, R=H) bilirubin dimethyl ester (I, R=CH_3) and VBA methyl ester (II)*

Mesobilirubin is similar to bilirubin, except for the vinyl groups ($C'_{(3)}$ and $C'_{(18)}$), which are replaced with ethyl groups.

Experimental

Materials

Bilirubin was obtained from Sigma. Bilirubin dimethyl ester and VBA methyl ester were prepared and purified as described previously (Plieninger *et al.*, 1972; Kaplan & Navon, 1981*a*). [^{2}H]DMSO, [^{2}H]acetone and [^{2}H]chloroform were from Merck.

For measurements where anhydrous samples were required, the pigments were dried over P_2O_5 in a vacuum for 24h at about 50°C. The solvent ([^{2}H]DMSO) was dried by repetitive treatments with molecular sieves 4A (obtained from BDH). A fresh portion of the drying agent, which had been pre-activated at a temperature of 500°C, was used in each treatment. The samples for n.m.r. spectroscopy were prepared by injecting the dry solvent, using a syringe purged with argon, into a septum-capped 5mm n.m.r. tube containing the dried pigment.

Methods

Most of the n.m.r. spectra were obtained on a Bruker WH-90 pulsed Fourier-transform spectrometer equipped with an Aspect 2000 computer. Spin-decoupled spectra were measured on the WH-300 spectrometer of The Hebrew University in Jerusalem.

Titrations of [^{2}H]chloroform solutions of bilirubin dimethyl ester with [^{2}H]DMSO were done by addition of a solution of the compound in [^{2}H]DMSO to the [^{2}H]chloroform solution and monitoring the chemical shifts of the protons as a function of the solvent composition. Internal tetramethylsilane was used as a reference, and the concentration of the ester (50mg/ml) and the temperature (26.0°C) were maintained constant.

The measurements of the selective and non-selective relaxation times, NOE and transfers of saturation were done as described previously (Kaplan & Navon, 1981*a*).

Results

Assignments of the n.m.r. spectra

Partial assignments of the spectra of the compounds in DMSO solutions have been published (Kuenzle, 1970; Maruyama, 1978; Kaplan & Navon, 1980, 1981*b*). In the present study, we assigned the resonances of the NH and methyl protons on the basis of our previous assignment of these resonances in [^{2}H]chloroform solutions (Kaplan & Navon, 1981*a*) and NOE experiments. The signals of the vinyl protons were assigned by spin-decoupling experiments at 300MHz and the spectral parameters were obtained by a simulation of the spectra. The complete assignment of the spectra and the spectral parameters of the vinyl protons are given in Tables 1 and 2 respectively.

Bilirubin and bilirubin dimethyl ester. The chemical shifts of most of the protons are similar for the two compounds. ^{2}H exchange experiments with [^{2}H]methanol revealed that the NH protons with chemical shifts of 9.92 and 10.05 p.p.m. exchange faster than the NH protons with chemical shifts of 10.47 and 10.49p.p.m. Hence the former two signals belong to the lactam protons and the latter pair belong to the pyrrole protons. This finding was confirmed by NOE measurements, where the irradiation of the signal of the methylene protons attached to $C_{(10)}$ produced an NOE on the signals of the pyrrole NH protons and vice versa (see below). The present assignment of the signals of the lactam and pyrrole NH protons is different from the assignment proposed by Kuenzle (1970).

The specific assignments of the signals of the lactam and methyl protons (Table 1) were obtained from a titration of a solution of bilirubin dimethyl ester with [^{2}H]DMSO (see the Experimental section). The titration curves are shown in Fig. 2. The resulting assignment of the signals of the methyl protons is in agreement with the assignment reported previously (Kaplan & Navon, 1980), which was based on a comparison with the spectrum of VBA.

The general behaviour of the chemical shifts of the NH and methyl protons of bilirubin dimethyl ester shows that a major change occurs around a mole fraction of DMSO of about 0.15. This change reflects the dissociation of the dimer as well as the solvation by DMSO. The similarity of the chemical shifts of these protons in bilirubin and its dimethyl

Table 1. *Assignments of proton spectra of bilirubin, bilirubin dimethyl ester and VBA methyl ester in [^2H]DMSO*
Sample concentrations were about 0.05, 0.14 and 0.15 M respectively. The spectra were obtained at 300 MHz at a temperature of 25°C. Chemical shifts are downfield from tetramethylsilane. Abbreviations used: BR, bilirubin; BRME, bilirubin dimethyl ester.

Bilirubin and bilirubin dimethyl ester[a]			VBA methyl ester		
δ (p.p.m.)	Assignment	Ref.	δ (p.p.m.)	Assignment	Ref.
1.93 (3H)	CH_3 group on $C_{(2)}$	b, c, d,	1.92 (3H)	CH_3 group on $C_{(2)}$	b
2.00 (3H), 2.04 (3H)	CH_3 groups on $C_{(7)}$ and $C_{(13)}$	b, c, d,	2.02 (3H)	CH_3 group on $C_{(7)}$	b
2.17 (3H)	CH_3 group on $C_{(17)}$	b, c, d			
2.22 (BR), 2.19 (BRME)	Centre of CH_2–CH_2 multiplet	e, f	2.56	Centre of CH_2–CH_2 multiplet	b, f
3.44 (6H)	Ester OCH_3	e	3.59 (3H)	Ester OCH_3	b
4.00 (2H)	CH_2 protons on $C_{(10)}$	e			
5.28–6.87 (m, 6H)	Vinyl groups	g	5.61–6.87 (m, 3H)	Vinyl group	g
6.09 (2H)	–CH= protons on $C_{(5)}$ and $C_{(15)}$	e	6.10 (1H)	–CH= proton on $C_{(5)}$	b
			6.78 (1H)	Proton on $C_{(9)}$	b
9.92 (1H) (BR), 9.88 (1H) (BRME)	Lactam NH proton on $N_{(24)}$	b			
10.00 (1H)	Lactam NH proton on $N_{(21)}$	b	9.97 (1H)	Lactam NH proton on $N_{(21)}$	b
10.47, 10.49 (2H)	NH protons on $N_{(22)}$ and $N_{(23)}$	b	10.60 (1H)	NH proton on $N_{(22)}$	b
11.89 (2H)	CO_2H protons	e			

[a] Unless mentioned otherwise, chemical shifts are identical for bilirubin and bilirubin dimethyl ester.
[b] The present work.
[c] Kaplan & Navon (1980).
[d] Maruyama (1978).
[e] Kuenzle (1970).
[f] The signals of the propionic CH_2–CH_2 protons are partially buried under the signals of the CH_3 groups and the broad [$^2H_5$]DMSO signal.
[g] For a complete assignment and spectral parameters, see Table 2.

Table 2. *Spectral parameters of vinyl protons in bilirubin, bilirubin dimethyl ester and VBA methyl ester*[a]
Chemical shifts are in p.p.m. downfield from tetramethylsilane; spin–spin coupling constants are in Hz and are accurate to ±0.5 Hz.

	δ_A	δ_B	δ_X	J_{AB}	J_{AX}	J_{BX}
exo ($C'_{(18)}$) vinyl	5.31	6.21	6.57	2.3	11.0	16.5
endo ($C'_{(3)}$) vinyl	5.66	5.65	6.82	2.1	10.5	17.0

[a] For experimental details see Table 1. The vinyl groups appear as ABX systems, where –CH= is X, H *cis* to the –CH= proton is A and H *trans* to the –CH= proton is B. The assignment of the signals to the exo and endo groups is according to Kaplan & Navon (1981*b*).

ester indicates that the two compounds are similarly solvated by DMSO.

The chemical shifts of the NH and CO_2H protons of bilirubin were found to be slightly changed when water was present in the solution. Thus the signals of the NH and CO_2H protons in a 0.05 M solution of bilirubin containing about 0.01 M-water were shifted downfield by 0.04 and 0.02 p.p.m. respectively, relative to their values in a dry solution at the same temperature.

VBA methyl ester. Specific assignments of the NH signals in the spectrum of this compound were obtained by spin-decoupling experiments, where an irradiation of the resonance of H of $C_{(9)}$ appearing at 6.78 p.p.m. caused a narrowing of the resonance of the pyrrole NH proton. The assignments of the

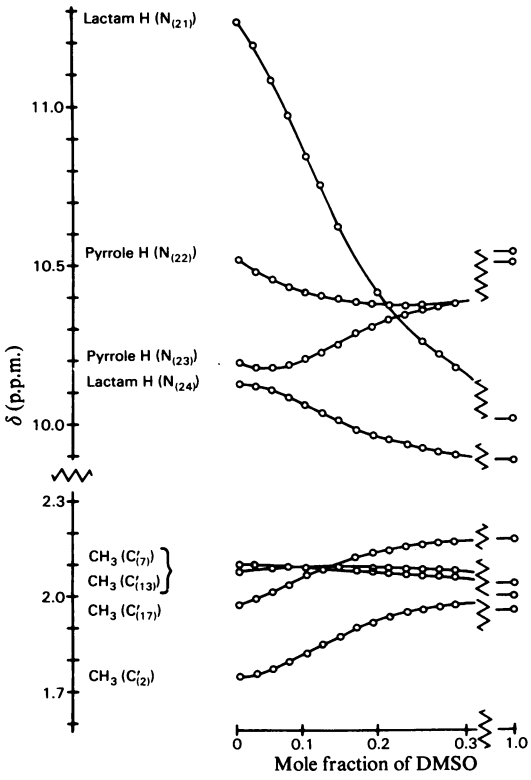

Fig. 2. *Correlations between the chemical shifts of NH and methyl protons of bilirubin dimethyl ester in chloroform and DMSO*

signals of the methyl protons were verified by NOE measurements, where an NOE was produced on the signal of the methine proton by irradiation at the signal of the $C'_{(7)}$ methyl group, whereas the irradiation at the resonance of the $C'_{(2)}$ methyl group did not produce any NOE. The state of solvation of VBA methyl ester seems to be similar to that of bilirubin and bilirubin dimethyl ester, as indicated by the similar chemical shifts in the spectra of these compounds.

Spin-lattice relaxation times, exchange processes and nuclear Overhauser enhancements

The main features of the conformation of bilirubin in solution are the angles between the planes of the pyrrolenone and pyrrole rings within each of the pyrromethenone fragments, the angles of rotation of these fragments about the central bridge carbon ($C_{(10)}$) and the orientation of the propionic acid residues relative to other parts of the molecule. These features may be expressed in terms of the distances between the protons of the lactam, pyrrole NH and carboxylic acid groups and their nearest neighbours. In general, the average distance between two protons, r_{ij}, can be evaluated from the contribution of their mutual dipolar interaction to the rates of spin-lattice relaxation. If the condition of extreme narrowing holds, i.e. if $\omega\tau_{ij} \ll 1$, where ω is the resonance frequency in rad/s and τ_{ij} is the rotational correlation time modulating the dipolar interaction between the protons i and j, r_{ij} can be calculated from the relation:

$$\langle r_{ij}^6 \rangle = 0.5\gamma_H^4 \hbar^2 \, T_1^s(i)' \, \tau_{ij}/\eta_i(j) \qquad (1)$$

(Noggle & Schirmer, 1971; Freeman *et al.*, 1974; Hall *et al.*, 1979), where $T_1^s(i)$ is the selective T_1 of proton i and $\eta_i(j)$ is the NOE produced on the resonance of i by irradiating the resonance of proton j. The relation between the selective T_1, non-selective T_1 (T_1^{ns}) and $\eta_i(j)$ is predicted by theory to be:

$$T_1^s(i)/T_1^{ns}(i) = 1 + \sum_{j \neq i} \eta_i(j) \qquad (2)$$

where the summation is carried over all the protons that interact dipolarly with i. Eqns. (1) and (2) are valid in the absence of three-spin effects (Noggle & Schirmer, 1971), as is the case in the present system. If the relaxation is dominated by proton–proton dipolar interactions, the sum over j of $\eta_i(j)$ is equal to 0.5.

If the observed proton exchanges chemically and the exchange rate is of the order of T_1, the recovery of the longitudinal magnetization becomes dependent on the exchange rate, especially in selective T_1 measurements (Campbell *et al.*, 1978). A quantitative comparison between the rate of exchange and the rate of spin-lattice relaxation in the absence of exchange may be done through saturation transfer experiments (Gupta & Redfield, 1970; Cheshnovsky & Navon, 1978).

In the NOE measurements involving the NH protons of bilirubin and its dimethyl ester, the partial overlap between the two lactam signals was removed by multiplying the free induction decay signal by a Lorentian-Gaussian exponential function before Fourier transformation. The extensive overlap between the signals of the pyrrole NH protons could not be removed. For these protons the errors in the NOE values were relatively large, and not all of these values were determined quantitatively.

The spin-lattice relaxation times and nuclear Overhauser enhancements for the protons of bilirubin, bilirubin dimethyl ester and VBA methyl ester are given in Table 3. These results, and their dependence on proton-exchange processes in the different compounds, are discussed below.

Bilirubin. The T_1^{ns} value of the bridge CH_2 protons attached to $C_{(10)}$ is very short, a result that is expected since this group belongs to the backbone of the molecule and its motional freedom is limited (Kaplan & Navon, 1981*b*). This T_1^{ns} value is shorter

Table 3. *Spin-lattice relaxation times (s) and nuclear Overhauser enhancements of protons of bilirubin, bilirubin dimethyl ester and VBA methyl ester in [^2H]DMSO solutions*[a]

	Observed resonance	Relaxation times of observed protons T_1^{ns}	T_1^s	$\Sigma\eta$ (from T_1^s/T_1^{ns})	Irradiated resonance	NOE on observed resonance
Bilirubin	CH_2 protons on $C_{(10)}$	0.080			$H(N_{(22)})$ and $H(N_{(23)})$	0.03
	$H(N_{(22)})$ and $H(N_{(23)})$	0.11	0.13[b]	0.18	$H(N_{(21)})$	c
	$H(N_{(22)})$ and $H(N_{(23)})$				$H(N_{(24)})$	c
	$H(N_{(22)})$ and $H(N_{(23)})$				CH_2 protons on $C_{(10)}$	0.06[b]
	$H(N_{(21)})$	0.153	0.18[b]	0.18	$H(N_{(22)})$ and $H(N_{(23)})$	0.16
	$H(N_{(24)})$	0.154	0.18[b]	0.17	$H(N_{(22)})$ and $H(N_{(23)})$	0.15
Bilirubin dimethyl ester	CH_2 protons on $C_{(10)}$	0.083				
	$H(N_{(22)})$ and $H(N_{(23)})$	0.11	0.14[b]	0.27	$H(N_{(21)})$	c
	$H(N_{(22)})$ and $H(N_{(23)})$				$H(N_{(24)})$	c
	$H(N_{(22)})$ and $H(N_{(23)})$				CH_2 protons on $C_{(10)}$	0.05[b]
	$H(N_{(21)})$	0.146	0.17[b]	0.16	$H(N_{(22)})$ and $H(N_{(23)})$	0.16
	$H(N_{(24)})$	0.144	0.17[b]	0.18	$H(N_{(22)})$ and $H(N_{(23)})$	0.16
VBA methyl ester	$H(N_{(22)})$	0.305	0.390	0.28	$H(N_{(21)})$	0.18
	$H(N_{(22)})$				$H(C_{(9)})$	0.08
	$H(N_{(21)})$	0.42	0.49	0.17	$H(N_{(22)})$	0.21
	$H(C_{(5)})$	0.57	0.79[d]	0.39	CH_3 proton (C_7')	0.18
	$H(C_{(5)})$				vinyl $-CH=$ proton	0.14[d]

[a] Obtained at a resonance frequency of 90 MHz. The concentrations in the solutions of bilirubin, bilirubin dimethyl ester and VBA methyl ester were 0.045, 0.14 and 0.15 M respectively. The random errors in the T_1 and NOE values were $\pm 10\%$ unless specified otherwise.

[b] $\pm 15\%$.

[c] A positive NOE was observed but no quantitative measurement was made.

[d] Value may be slightly underestimated (see the text).

than the corresponding T_1^{ns} value in chloroform solutions by a factor of about 3.5, which reflects the corresponding change in the rotational correlation time of the molecule (Kaplan & Navon, 1981*a*). As expected, the NOEs observed between the bridge CH_2 protons and the pyrrole NH protons are small, owing to predominant dipolar interactions between the geminal protons of the CH_2 group and $^1H-^{14}N$ dipolar interaction in the pyrrole NH groups. Similar results have been observed for mesobilirubin and bilirubin dimethyl ester in chloroform solutions (Kaplan & Navon, 1981*a*).

The observation of an NOE between exchangeable protons is usually difficult because of the process of saturation transfer, which has an opposite effect on the intensity of the observed signal. In preliminary experiments with [^2H]DMSO solutions that contained about 0.02M-water, the saturation of the water signal caused a decrease in the CO_2H signal to about 10% of its value (a saturation transfer of 0.9). The saturation of the CO_2H signal caused a saturation transfer of about 0.7 to the water signal. In both of the above experiments, the signals of the NH protons were not affected. The irradiation of the signal of the pyrrole NH protons had no influence on either the CO_2H or the water signals. However, the irradiation of the signals of the lactam protons (on $N_{(21)}$ and $N_{(24)}$) produced, in addition to the NOE on the pyrrole NH signals, saturation transfers of about 0.07 and 0.03 respectively, to the signals of the CO_2H protons. The reason that the saturation of the carboxylic acid resonance does not affect the lactam resonances is the shorter T_1 of the lactam protons.

To be able to detect an NOE between the carboxylic acid and lactam protons of bilirubin, an effort was made to minimize the exchange rates by working with dry solutions. Thus solutions of bilirubin were prepared with [^2H]DMSO that had been dried extensively (see the Experimental section) and were transferred to a dried n.m.r. sample tube, which contained a few pellets of molecular sieves 4A. No water signal could be observed in the n.m.r. spectrum of these solutions, indicating that the molar concentration of water was less than about one-fiftieth of the molar concentration of bilirubin, i.e. less than 0.001 M. The spectra obtained from these dried solutions (Fig. 3*a*) are distinguished from spectra of bilirubin published previously (Kuenzle, 1970; Chedekel *et al.*, 1974; Navon, 1976; Kaplan & Navon, 1980) in that the signal of the CO_2H proton is fairly narrow. Although the rate of exchange between the CO_2H protons and water was still of the order of T_1 (as shown by the saturation

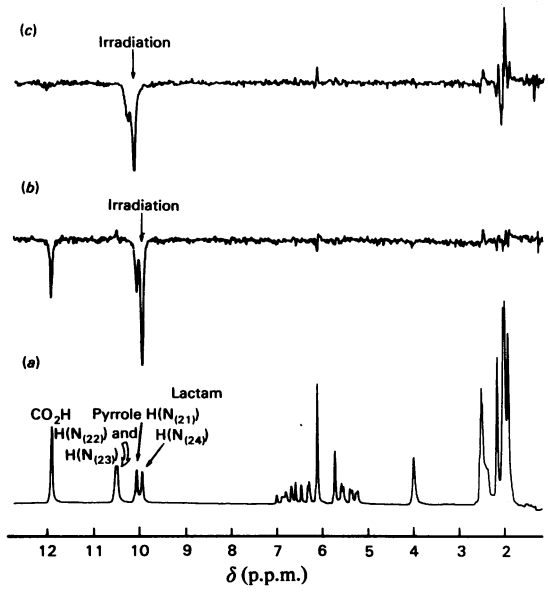

Fig. 3. *90 MHz ^{1}H-n.m.r. spectra of bilirubin in thoroughly dried solutions*

(*a*) Spectrum in [^{2}H]DMSO, at 30°C (for the complete assignment, see Tables 1 and 2). (*b*) Double-irradiation difference spectrum showing a saturation transfer from the lactam to the CO_2H signals and an NOE on the pyrrole NH signals. Sample and temperature are the same as in (*a*). (*c*) Double-irradiation difference spectrum in [^{2}H]DMSO/[^{2}H]acetone (1:1) at −0.4°C. The NOE on the pyrrole NH signals is annihilated by direct saturation, the difference in chemical shift between the lactam and pyrrole NH signals being smaller by about 5 Hz than for [^{2}H]DMSO solution at 30°C.

transfer to the CO_2H signal on irradiating the position of the water resonance), the low concentration of water made this exchange unimportant with respect to the relaxation of the carboxylic acid protons. Unexpectedly, the saturation transfer between the lactam and CO_2H resonances (Fig. 3*b*) was found to be larger in the presence of the molecular sieves. The saturation transfer from the lactam to the CO_2H signals reached an average value of 0.23. Also, a saturation transfer in the reverse direction could be observed in the dry solutions, its values being about 0.06 and 0.02 for the signals of the protons $N_{(21)}$ and $N_{(24)}$ respectively. This result is not due to changes in T_1, as the T_1^{ns} values of the lactam protons in the absence of exchange, measured by the method of Mann (1977), were found to be identical, within experimental error, with the values obtained from non-dried solutions. The increased saturation transfer between the lactam and CO_2H resonances in the presence of molecular sieves thus indicates that the rate of proton exchange between these sites is catalysed in the presence of molecular sieves, possibly due to some basic impurities.

Further experiments were done on dried solutions of bilirubin in a mixture of [^{2}H]DMSO and [^{2}H]acetone, 1:1 (v/v), at low temperatures. At temperatures of −0.4°C and −4·5°C, the saturation of the lactam resonances did not change the intensity of the CO_2H resonance (Fig. 3*c*), and the saturation of the CO_2H signal had no effect on the intensity of the lactam signals. Since rates of proton exchange are usually dependent on the temperature, whereas the NOE is not, it follows from the results above that there is no cancellation of significant effects of saturation transfer and NOE, but rather that both are smaller than the experimental error of our measurements, i.e. smaller than about 0.02. The above results probably apply also to solutions of bilirubin in pure [^{2}H]DMSO at room temperature, for the following reasons. (*a*) The T_1^{ns} values of the bridge CH_2 and pyrrole NH protons in the [^{2}H]DMSO/[^{2}H]acetone solution at −0.4°C are 0.096 and 0.12s (±10%) respectively, and are thus fairly similar to the corresponding values in [^{2}H]DMSO solutions at 30°C (Table 3). Hence the rotational correlation times of the molecule are similar in the two experiments. (*b*) The proton chemical shifts of bilirubin in the solutions containing [^{2}H]acetone are similar, within ±0.05 p.p.m. to the corresponding chemical shifts of the compound in pure [^{2}H]DMSO. Thus the presence of moderate concentrations of acetone has caused no significant change in the conformation of the molecule.

The NOEs between the lactam and pyrrole NH protons (Table 3) indicate that the two fragments of the molecule have a *syn-Z* structure. As expected, the values of $\Sigma\eta$ for the lactam protons are considerably smaller than 0.5, as a result of the contributions of the dipolar interaction with the ^{14}N nuclei to the relaxation of these protons. The agreement between the values of the NOE produced on the lactam signals by irradiating the pyrrole NH signals and the values of $\Sigma\eta$ is consistent with the absence of any NOE between the lactam protons and other protons in the molecule. However, this result should not be over-emphasized because of the relatively large experimental error in the values of $\Sigma\eta$.

Bilirubin dimethyl ester and VBA methyl ester. Saturation transfer experiments with non-dried solutions of the two compounds in [^{2}H]DMSO indicated that the proton exchange between the lactam and pyrrole NH groups and water is very slow compared with T_1, as is the case for bilirubin. The NOE between the lactam and pyrrole NH protons of bilirubin dimethyl ester and VBA methyl ester, and between the $C'_{(7)}$ methyl protons and the

proton of $C_{(5)}$ of VBA methyl ester (Table 3) established the *syn-Z* structure about the methine bonds in the two compounds.

For VBA methyl ester, a comparison of $\Sigma\eta$ for the NH protons, obtained from the values of their T_1^s and T_1^{ns}, with the sum of NOE values obtained by the irradiation of the various neighbouring protons indicates that the interaction with the pyrrole NH proton accounts for the homonuclear dipolar relaxation of the lactam proton. For the pyrrole NH proton, the homonuclear dipolar contribution to the relaxation is provided by the lactam proton and by the proton attached to $C_{(9)}$. For the methine proton on $C_{(5)}$, the set of NOE values in Table 3, i.e. the NOE with the methyl group on $C_{(7)}$ and the vinyl $-CH=$ proton, is probably incomplete, since the proton on $C_{(5)}$ may interact also with one of the vinyl $CH_2=$ protons. However, the proximity of the resonance of the vinyl protons, especially those of the $CH_2=$ group, interfered with the measurement of NOE and selective T_1 of the proton on $C_{(5)}$. This may be also the reason for the value of $\Sigma\eta$ being smaller than the theoretical value of 0.5.

Deuteration experiments. The exchange of the labile protons in bilirubin and its derivatives with [^{2}H]methanol was monitored by recording the intensity of their signals as a function of time. The CO_2H, lactam and pyrrole NH protons of bilirubin, and the lactam and pyrrole NH protons of bilirubin dimethyl ester and VBA methyl ester were found to be completely exchanged within 1 min.

Interproton distances in bilirubin and bilirubin dimethyl ester

^{13}C relaxation studies have shown that the re-orientation of the backbone of the molecules of bilirubin and its dimethyl ester in [^{2}H]DMSO is determined by a single isotropic correlation time, τ_R (Kaplan & Navon, 1981*b*). The calculation of interproton distances in the present work is based on the assumption that the values of τ_{ij} are approximately equal to τ_R.

The distances between each of the pyrrole NH protons and the two bridge CH_2 protons were calculated from eqn. (1) by using the T_1^s and NOE data of the pyrrole NH protons. The determination of the separate contributions of $H_{(1)}$ on $C_{(10)}$ and $H_{(2)}$ on $C_{(10)}$ to the dipolar relaxation of protons on $N_{(22)}$ and $N_{(23)}$ was done on the basis of the relative spatial positions of these protons as derived from the X-ray data of bilirubin (Le Bas *et al.*, 1980), by analogy with the calculations of the interproton distances in mesobilirubin (Kaplan & Navon, 1981*a*).

The interproton distances calculated for bilirubin and bilirubin dimethyl ester in DMSO solutions are given in Table 4. For comparison, the corresponding interproton distances in mesobilirubin in chloroform solutions and in crystalline bilirubin are also included in Table 4. The conformations of bilirubin and mesobilirubin in chloroform are identical as shown by the n.m.r. data reported previously (Kaplan & Navon, 1981*a*). The distances between each of the pyrrole NH protons and its nearest neighbouring proton on $C_{(10)}$ in DMSO are similar to the corresponding distances in chloroform solutions. The distances between each lactam proton and the pyrrole NH proton of the same pyrromethenone fragment in DMSO are larger by 10% than the corresponding values in mesobilirubin in chloroform.

The n.m.r. relaxation data obtained in the present study are not sufficient for an estimation of the position of the CO_2H protons of bilirubin relative to other protons of the molecule. However, a lower limit for the distance between each lactam proton and the nearest CO_2H proton can be made on basis of the T_1^s values of the lactam protons and the

Table 4. *Interproton distances (nm) in bilirubin and its derivatives in solution*

	Solvent	$10^{10} \times \tau_R$ (s)[a]	Pyrrole NH–nearest H on $C_{(10)}$	$H(N_{(21)})$–$H(N_{(22)})$	$H(N_{(23)})$–$H(N_{(24)})$	Lactam NH–nearest CO_2H
Bilirubin[b]	DMSO	2.2	0.23	0.21	0.21	>0.28
Bilirubin dimethyl ester[b]	DMSO	2.4	0.24	0.21	0.21	—
Mesobilirubin[c]	Chloroform	0.59	0.24	0.19	0.19	0.23
Crystalline bilirubin	—	—	0.28[e]	0.154[d]	0.158[d]	0.25[e]
	—	—	0.25[f]	0.191[f]	0.187[f]	0.22[f]

[a] Obtained from ^{13}C T_1 data (Kaplan & Navon, 1981*b*).
[b] Present work.
[c] Kaplan & Navon (1981*a*).
[d] Bonnett *et al.* (1978).
[e] Calculated from the atomic parameters reported by Bonnett *et al.* (1978).
[f] Calculated from the atomic parameters reported by Le Bas *et al.* (1980).

experimental error of 0.02 for the NOE between these protons. The lower limit for the lactam proton–CO_2H proton distance thus obtained is 0.28nm.

Discussion

The chemical shifts of the NH and COOH protons of bilirubin and related compounds in DMSO solutions are expected to be affected by intramolecular hydrogen-bonding or by hydrogen-bonding with the solvent. In fact, DMSO is known to form hydrogen bonds with the NH group of amides (La Planche *et al.*, 1965; Porter & Brey, 1967; Tewari *et al.*, 1971), pyrrole (Spencer *et al.*, 1978), indoles (Jardine & Brown, 1963) and carboxylic acids (Chantooni & Kolthoff, 1975). The chemical shifts of the lactam and pyrrole NH protons of bilirubin and its derivatives (Table 1) are typical for NH protons in DMSO solutions. Moreover, the fact that the chemical shifts of the lactam and pyrrole NH protons of bilirubin, bilirubin dimethyl ester and VBA methyl ester (Table 1), as well as VBA (Kaplan & Navon, 1980), are very similar, is evidence that these chemical shifts are determined by hydrogen-bonding with the solvent rather than intramolecular interactions, since in VBA and its methyl ester no internal hydrogen-bonding is possible. Similar conclusions can be drawn for the propionic CO_2H protons of bilirubin since their chemical shift ($\delta = 11.89$ p.p.m.) is similar to that of VBA [$\delta = 12.07$ p.p.m. (Kaplan & Navon, 1980)] but quite different from that of bilirubin in chloroform solutions [$\delta = 13.7$ p.p.m. (Manitto *et al.*, 1974; Kaplan & Navon, 1981*a*)].

Two experimental facts indicate that the hydrogen-bonds between the propionic acid residues and the pyrrole and lactam NH groups, which occur in chloroform solutions, do not exist in DMSO solutions. (*a*) The rate of proton exchange of the pyrrole NH groups of bilirubin with [^{2}H]methanol is faster than $2 \times 10^{-2} s^{-1}$ in DMSO, i.e. more than three orders of magnitude as fast as in chloroform containing a similar concentration of [^{2}H]methanol (Kaplan & Navon, 1981*a*). (*b*) The distance between the lactam and CO_2H protons of bilirubin in DMSO is larger by at least 0.05nm than the corresponding distances in mesobilirubin in chloroform solutions.

The interproton distances calculated from the present n.m.r. data for bilirubin and its derivatives in DMSO solutions indicate that the pyrromethenone fragments of these molecules have a *syn-Z* structure, as they do in chloroform solutions (Kaplan & Navon, 1981*a*) and in the crystalline state (Bonnett *et al.*, 1978; Becker & Sheldrick, 1978). The distance between each pyrrole NH proton and the nearest bridge CH_2 proton on $C_{(10)}$ is relatively short

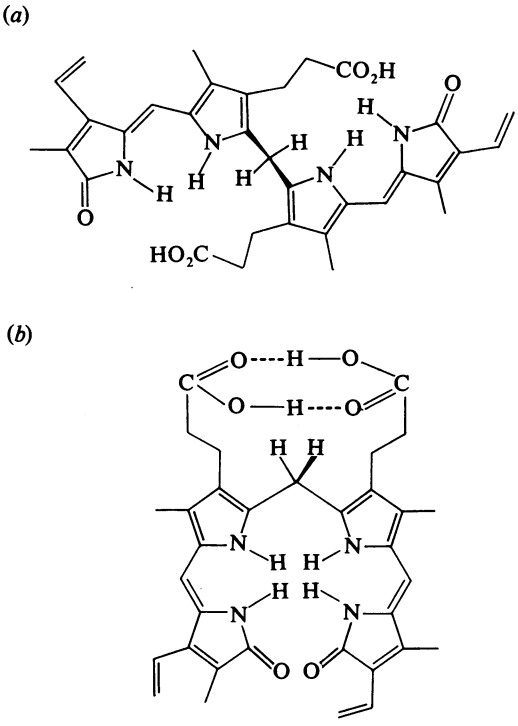

Fig. 4. *Two of the possible conformations of bilirubin in DMSO solutions*

Note that in (*a*), each pyrrole NH proton lies close to one of the central bridge CH_2 protons, whereas in (*b*), each pyrrole NH proton is equidistant from the two CH_2 protons.

and indicates an extended conformation of the whole molecule (Fig. 4*a*) similar to that found in the crystalline state and in chloroform solutions. These distances rule out completely the conformation shown in Fig. 4(*b*), which is similar to the structures suggested for bilirubin by Nichol & Morell (1969) and by Hutchinson *et al.* (1971) and, for bilirubin dimethyl ester, by Manitto *et al.* (1974). In this structure (Fig. 4*b*) the pyrrole NH–H on $C_{(10)}$ distance is large (about 0.38nm) and the NOE between these protons would be negligibly small.

Further information about the conformation of the central dipyrrylmethane fragment of bilirubin and its dimethyl ester may be obtained from a comparison of the values of the NOE produced on the pyrrole NH signals by irradiating the bridge CH_2 signal. These values are about the same in bilirubin and its dimethyl ester in DMSO and in mesobilirubin in chloroform solutions (Kaplan & Navon, 1981*a*). Thus the orientation of the pyrrole groups relative to the central CH_2 group is probably similar in all of the above cases.

The significance of the 10% increase in the value of the lactam–pyrrole NH interproton distance in bilirubin in DMSO relative to its value in chloroform deserves some consideration. Such a difference corresponds to a difference of 77% in the ratio $T_1^s(i)\,\tau_{ij}/\eta_i(j)$ (eqn. 1). Although the combined experimental errors in the values of $T_1^s(i)$ and $\eta_i(j)$ given in Table 3 are much smaller than 77%, one cannot ignore the assumptions involved in obtaining the values of τ_{ij} in DMSO and in chloroform (Kaplan & Navon, 1981*a*). In both cases, these values are based on the rotational correlation times of the backbone derived from ^{13}C T_1 values. Thus any extra motion of the NH groups may affect the τ_{ij} values. On the other hand, a slight increase in the lactam–pyrrole NH interproton distances in DMSO solution is in line with the solvation, described above in this section, of the NH groups by DMSO, owing to the steric repulsion between the solvating molecules. Thus the larger lactam–pyrrole NH distances in DMSO may indicate a slightly non-planar structure of each of the pyrromethenone fragments.

Our previous ^{13}C relaxation studies have shown that the motional freedom of the propionic side-chain of bilirubin, bilirubin dimethyl ester and mesobilirubin dimethyl ester in DMSO solutions is very limited (Kaplan & Navon, 1981*b*). This is in contrast with the independent fast motion of the propionic residue of VBA methyl ester, where the structure does not allow internal hydrogen-bonding. The possibility of direct hydrogen bonding between the propionic residues and the pyrrole and lactam NH groups of bilirubin and its dimethyl ester is invalidated by the large lactam NH–CO_2H distance and by the faster exchange of the pyrrole NH protons. We thus conclude that the propionic CO_2H or CO_2CH_3 residues are tied to their nearest pyrrole NH and lactam groups (see Fig. 4*a*) via bound solvent molecules.

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