Nuclear Magnetic Resonance Studies of the Conformation of Bilirubin and its Derivatives in Solution

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Selective and non-selective spin–lattice relaxation times (T_1), nuclear Overhauser enhancements (NOE), and rates of exchange of protons in bilirubin and related compounds in chloroform solutions were measured. The NOE observed between methyl and methine protons and between lactam and pyrrole NH protons indicate that the configuration within the pyrromethenone units is exclusively *syn-Z*. An NOE was also observed between the protons of the carboxylic acid and the lactam NH groups of mesobilirubin. The selective T_1 relaxation times, NOE values of the protons, and correlation times calculated from carbon-13 relaxation data were used for the calculation of inter-proton distances. These distances, as well as the extremely slow rates of exchange of the pyrrole NH protons, establish the presence of hydrogen bonds between the carboxy-residues and the lactam and pyrrole NH groups of mesobilirubin in chloroform solutions. The T_1 values, chemical shifts, and rates of proton exchange in bilirubin are similar to those of mesobilirubin, indicating that the same conformation occurs for the two compounds. The conformation of bilirubin dimethyl ester is very different from that of bilirubin and mesobilirubin. It is present as a dimer even at concentrations as high as 0.17M. The proton exchange rates of both the lactam and pyrrole NH groups of bilirubin dimethyl ester are slightly slower than in a model pyrromethenone compound.

KNOWLEDGE of the conformation of bilirubin is important for the understanding of its metabolism, in particular in jaundice which may lead to kernicterus in newborn infants, and of the mechanism of phototherapy.[1] This has stimulated several investigations of its chemical and spectroscopic properties in the last two decades. As a consequence, different conformations were suggested for bilirubin and its dimethyl ester in solution.

On the basis of i.r. spectra of bilirubin and partially deuteriated bilirubin in chloroform solutions, Brodersen *et al.*[2] suggested that the carboxylic acid groups of the 8- and 12-propionic side-chains (see Figure 1) are hydrogen-bonded to the pyrrole protons 23- and 22-H, respectively.

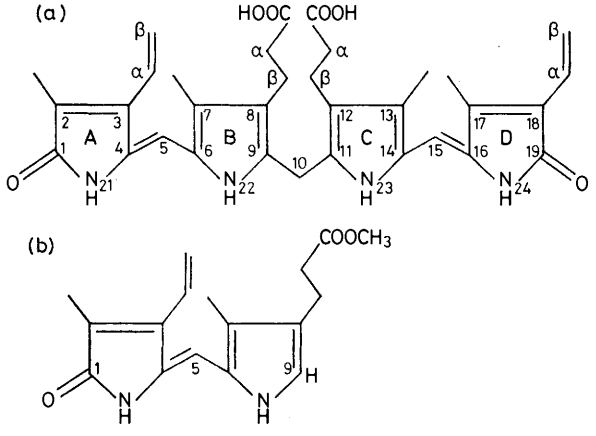

FIGURE 1 Schematic structures of bilirubin (a) and vinyl-neoxanthobilirubinic acid methyl ester (b). Mesobilirubin differs from bilirubin in that the 3- and 18-vinyl groups are replaced with ethyl groups

Hutchinson *et al.*,[3] who also utilized i.r. spectroscopy and deuterium exchange of the labile protons, raised the possibility of another conformation involving hydrogen bonds between the two carboxy-groups and free, non-hydrogen-bonded NH groups. Kuenzle *et al.*[4] investigated the reaction of bilirubin with diazomethane, where derivatives with lactim ether groups are formed besides the simple dimethyl ester. These authors proposed a conformation for bilirubin in which the carboxy-groups are hydrogen-bonded to the lactam C=O and NH groups so that the structure is stabilized by four intramolecular hydrogen bonds. They also proposed that the two hydrogen bonds between the oxygen atoms of the propionic carbonyl groups and the lactam protons 21- and 24-H exist in one of the possible conformations of bilirubin dimethyl ester in chloroform solutions. The same pattern of the four internal hydrogen bonds suggested by Kuenzle *et al.* was independently proposed for bilirubin by Manitto *et al.*[5] on the basis of the i.r. spectra and n.m.r. chemical shifts of bilirubin and some derivatives. However, in contrast to Kuenzle *et al.*, these authors explained the chemical shifts of the ring methyl groups in the spectrum of bilirubin dimethyl ester as resulting from a non-hydrogen-bonded conformation with the two pyrromethenone units being juxtaposed to each other. In a later communication, Manitto and Monti [6] reported an n.m.r. indication for a temperature-dependent rotation of the two pyrromethenone units of bilirubin about their bonds with the central carbon, C-10, of the molecule. These authors interpreted their results in terms of six intramolecular hydrogen bonds, including the bonding of the COOH groups to both the lactam (as mentioned above) and the pyrrole NH groups. This conformation was recently found by Bonnett *et al.*[7] to occur for bilirubin in the crystalline state; a similar structure was found by Becker and Sheldrick [8] to occur in crystalline mesobilirubin. Carbon-13 relaxation measurements have indicated that bilirubin dimethyl ester is dimeric in chloroform solutions, and that its propionic side-chains are not hydrogen-bonded in this solvent.[9] The occurrence of the ester as a dimer was independently detected through the osmometric studies of Holzwarth *et al.*[10] and of the present authors.[9]

Nuclear Overhauser enhancements (NOE) and spin–

lattice relaxation times (T_1) may be used to monitor dipolar interactions between protons and thus to provide information about the internal geometry of the molecule.[11]

In this paper, we report an n.m.r. study of nuclear Overhauser effects, spin-lattice relaxation times, and rates of exchange of protons in bilirubin and some of its derivatives. Preliminary accounts of these results have been presented elsewhere.[9b]

EXPERIMENTAL

Materials.—Bilirubin was purchased from Sigma. Mesobilirubin was prepared by catalytic hydrogenation of bilirubin according to procedures given in the literature.[12,13] Vinylneoxanthobilirubinic acid methyl ester (VBA methyl ester), was prepared and purified as described in the literature.[9,12,14-16]

Bilirubin dimethyl ester was prepared by a slightly modified version of Kuenzle's procedure.[4] Esterification was performed on a suspension of bilirubin in dichloromethane, and the products were separated by chromatography on a column (45 × 2.5 cm) of alumina (Brockmann grade II—III). The eluants used were dichloromethane, chloroform (containing *ca.* 0.8% of ethanol), and chloroform–methanol (95 : 5 v/v) in that order. Bilirubin dimethyl ester was recovered from the chloroform–methanol eluate and was recrystallized from chloroform–methanol (1 : 2 v/v).

All the compounds mentioned above were shown to be pure by t.l.c. on silica gel, using two separate eluant systems [chloroform–ethyl acetate (3 : 1) and chloroform containing 1% of acetic acid] and all gave the expected ^{1}H n.m.r. spectrum. In particular, the 90-MHz n.m.r. spectrum of bilirubin dimethyl ester from ten different batches, dissolved in $CDCl_3$, was consistent with a single species. This result is in agreement with the recent report of Holzwarth *et al.*[10] but differs from the results of previous investigators.[4,13]

Solvents.—The deuteriated solvents used were purchased from E. Merck (Darmstadt). Deuteriochloroform was distilled from P_2O_5 under nitrogen prior to use. Methanol (AnalaR) was distilled from $CaSO_4$.

Deuteriation Products of Bilirubin Dimethyl Ester.—When [$^2H_4$]methanol is added to a solution of bilirubin dimethyl ester in $CDCl_3$, the NH signals at δ 10.10 and 11.23 in the ^{1}H n.m.r. spectrum disappear in <1 min whereas the other two protons are exchanged in *ca.* 6—8 min. On the basis of this experiment, we prepared dideuteriobilirubin dimethyl ester by dissolving bilirubin dimethyl ester (70 mg) in dry $CHCl_3$ (0.8 ml), adding quickly CD_3OD (0.12 ml) and, after agitating the solution for 1 min, removing the solvents within *ca.* 20 s by evaporation *in vacuo*. A tetradeuteriobilirubin dimethyl ester, in which all four NH protons are replaced with deuterons, was prepared by letting the reaction proceed for *ca.* 15 min before the evaporation step. The deuteriation products, designated by bilirubin dimethyl ester-$(NH)_2(ND)_2$ and bilirubin dimethyl ester-$(ND)_4$, respectively, were dried over P_2O_5 in a vacuum dessicator.

Methods.—Most ^{1}H n.m.r. spectra were obtained on a Bruker WH-90 pulsed Fourier-transform spectrometer equipped with an Aspect 2000 computer. The spin-decoupled ^{1}H spectra as well as the ^{13}C spectra were measured on the WH-300 spectrometer of the Hebrew University in Jerusalem.

The measurements of spin–lattice relaxation times and nuclear Overhauser enhancements were performed on both degassed samples, prepared by four freeze–pump–thaw cycles, and undegassed solutions. Degassing was found to have a negligible effect on the results except for the solution of mesobilirubin in chloroform, where values of T_1 longer than 1 s were observed.

Spin–lattice relaxation times were measured by the inversion–recovery technique. Selective T_1 measurements [17] of individual protons were performed by using a long (*ca.* 50 ms), weak pre-irradiation pulse at the resonance frequency of choice. The relaxation times were calculated from the experimental data by a two-parameter least-squares fitting of the initial part (τ 0—$0.7T_1$) of the decay curves. Each value of T_1 presented here is an average of two or three separate measurements.

Nuclear Overhauser enhancements (NOE) and transfer of saturation effects were measured by difference spectroscopy, using the 'NOE difference' microprogram of the Aspect 2000 computer. The waiting period for signal recovery was kept longer than 8 T_1. The enhancements were measured as a function of the intensity of irradiation and the values reported here are all in the plateau region of the function. In cases where the separation between the irradiated and the observed peaks was smaller than *ca.* 100 Hz, the effects of direct saturation on the peak area were eliminated by placing the on-resonance frequency and the off-resonance reference frequency symmetrically on both sides of the observed peak. The enhancements were calculated from the integrated intensities of the peaks. Integration was done manually, using a polar planimeter. The values of NOE presented here are the average of at least four separate measurements.

Unless otherwise stated, the T_1 and NOE measurements were performed at a temperature of 30.0 °C.

RESULTS AND DISCUSSION

I *Assignments of* ^{1}H *N.M.R. Spectra.*—Partial assignments of the spectra of bilirubin, bilirubin dimethyl ester, and VBA methyl ester in $CDCl_3$ have appeared in the literature.[5,13,18-20] In the present study, we assigned the vinyl proton signals in the spectra by homodecoupling experiments at 300 MHz. The spectral parameters were obtained by a simulation of the spectra, using the simulation program of the Aspect 2000 computer. The assignment of the NH, methine, and methyl resonances was achieved through deuterium exchange and NOE experiments as described below. The complete assignment of the spectra is given in Table 1. The spectral parameters of the vinyl protons are given in Table 2.

Bilirubin and mesobilirubin. The chemical shifts of the low-field signals are similar in the two compounds. The signal at δ *ca.* 13.7 belongs to the COOH protons. It disappears upon the addition of H_2O or when the sample temperature is raised above 50 °C, but is fairly sharp in a dry solution at a temperature of −20 °C (see Figure 2). The assignment of the NH signals was made on the basis of their different exchange rates with CD_3OD.

Bilirubin dimethyl ester. The four signals for the NH protons appear at δ 10.10, 10.18, 10.49, and 11.23. The peaks at δ 10.10 and 11.23 were assigned to the lactam

TABLE 1

Assignments of proton spectra of bilirubin, mesobilirubin, bilirubin dimethyl ester, and VBA methyl ester in $CDCl_3$. Sample concentrations were 0.01—0.03M, except for bilirubin which was *ca.* 0.0017M. The spectra were obtained at 300 MHz at 25 °C. Chemical shifts in p.p.m. are downfield from Me_4Si. Peaks are singlets unless stated otherwise: d = doublet, q = quartet, m = multiplet

Bilirubin			Mesobilirubin		
δ	Assignment	Ref.	δ	Assignment	Ref.
			1.03—1.13 (6 H, m)	CH_3 protons of ethyl groups on C-3 and -18	*a*
1.99 (3 H), 2.16 (6 H) 2.17 (3 H)	CH_3 groups	19	1.86 (3 H), 2.07 (3 H)	CH_3 groups	*a*
			2.15, 2.17 (6 H)		
			2.35 (2 H, q) 2.51 (2 H, q)	CH_2 protons of ethyl groups on C-3 and -18	*a*
2.54—3.05 (8 H, m)	CH_2–CH_2 protons of propionic sidechains	19	2.53—3.07 (8 H, m)	CH_2CH_2 protons of propionic side-chains	*a*
4.08 (2 H)	10-CH_2 protons	19	4.07 (2 H)	10-CH_2 protons	*a*
5.34—6.66 (m), 6.13, 6.20 (8 H)	vinyl groups	*a*			
	–CH= protons on C-5 and -15	19	6.05 (2 H)	–CH= protons on C-5 and -15	*a*
9.27, 9.30 (2 H)	NH protons on N-22 and -23	5, *a*	9.15 (2 H)	NH protons on N-22 and -23	*a*
10.70 (1 H), 10.80 (1 H)	lactam protons on N-21 and -24	5, *a*	10.61 (2 H)	lactam protons on N-21 and -24	*a*
13.69 (2 H)	COOH protons	5, *a*	13.66 (2 H)	COOH protons	*a*
Bilirubin dimethyl ester			VBA methyl ester		
1.75 (3 H)	2-CH_3 group	*a*	2.06 (3 H)	2-CH_3 group	*a*
1.98 (3 H)	17-CH_3 group	*a*	2.14 (3 H)	7-CH_3 group	*a*
2.08, 2.09 (6 H)	CH_3 groups on C-7 and -13	*a*			
2.48—2.89 (8 H, m)	CH_2CH_2 protons on propionic sidechains	13	2.10—3.25 (4 H, m)	CH_2CH_2 protons on propionic sidechain	*a*
3.70 (6 H)	ester CH_3	13	3.68 (3 H)	ester CH_3	*a*
4.17 (2 H)	10-CH_2 protons	13			
4.76—6.60 (6 H, m)	vinyl groups	*a*	5.61—6.74 (3 H, m)	vinyl group	*a*
5.90 (1 H)	–CH= proton on C-15	*a*			
6.20 (1 H)	–CH= proton on C-5	*a*	6.30 (1 H)	–CH= proton on C-5	20
			6.86 (1 H, d)	–CH= proton on C-9	20, *a*
10.10 (1 H)	lactam proton on N-24	*a*			
10.18 (1 H)	NH proton on N-23	*a*			
10.49 (1 H)	NH proton on N-22	*a*	10.48 (1 H)	NH proton on N-22	20, *a*
11.23 (1 H)	lactam proton on N-21	*a*	11.22 (1 H)	lactam proton on N-21	20, *a*

[a] Present work.

protons through the ^{13}C spectrum of bilirubin dimethyl ester-$(NH)_2(ND)_2$ as compared to the spectra of the non-deuteriated dimethyl ester and of bilirubin dimethyl ester-$(ND)_4$. These spectra (Figure 3) were measured at the same temperature, the same pulse angle, and the same delay time between pulses. In the spectrum of bilirubin dimethyl ester-$(NH)_2(ND)_2$, the peaks of the carbonyl carbons C-1 and C-19 are considerably attenuated and are shifted upfield by *ca.* 0.14 and 0.12 p.p.m., respectively. The attenuation of the signals results from an increase of their spin–lattice relaxation times and loss of ^1H–^{13}C NOE, and indicates that the lactam NH protons are replaced with deuterons. The peaks of the ring carbons C-4 and C-16, the assignment of which is based on the work of Hansen *et al.*[21] are also attenuated and are shifted by *ca.* 0.1 p.p.m. However, the peaks of C-9 and C-11 in the spectrum of bilirubin dimethyl ester-$(NH)_2(ND)_2$ are similar in position and intensity to their analogues in bilirubin dimethyl ester, indicating that in the former compound the pyrrole NH protons have remained mostly unexchanged. In the spectrum of bilirubin dimethyl ester-$(ND)_4$, the peaks of the above-mentioned six carbons are all attenuated and isotope shifted. Thus the ^1H peaks at δ 10.10 and 11.23, which disappear faster than the two other peaks upon deuterium exchange, are due to the lactam NH protons.

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 Me_4Si; spin–spin coupling constants are in Hz and are accurate to ±0.5 Hz

		Bilirubin	Bilirubin dimethyl ester	VBA methyl ester
exo-18-vinyl	δ_A	5.36	4.81	
	δ_B	6.16	5.28	
	δ_X	6.49	6.09	
	J_{AB}	2.0	1.5	
	J_{AX}	10.2	10.5	
	J_{BX}	17.5	17.3	
endo-3-vinyl	δ_A	5.60	5.46	5.65
	δ_B	5.59	5.53	5.63
	δ_X	6.61	6.56	6.68
	J_{AB}	1.9	1.6	1.5
	J_{AX}	11.2	12.1	11.0
	J_{BX}	17.8	17.1	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.

This assignment is different from that given by Manitto *et al.*[5]

When the peak of a lactam proton at δ 11.23 was irradiated strongly, an NOE of 0.12 was observed on the pyrrole peak at δ 10.49. The reverse effect, namely an NOE on the lactam peak upon irradiation of the pyrrole peak was also observed. This result implies that the

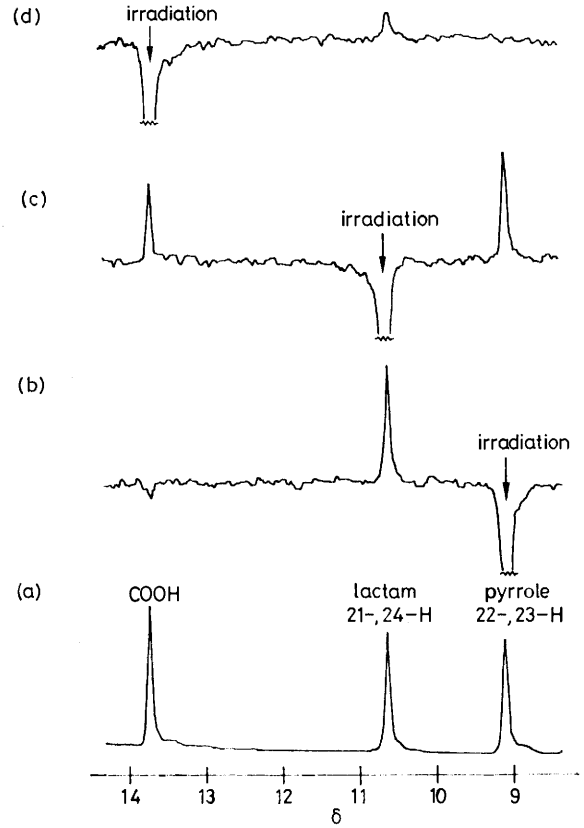

FIGURE 2 ^{1}H N.m.r. spectrum (a) and NOE difference spectra (b—d) of mesobilirubin in $CDCl_3$ at 90 MHz, at a temperature of −20 °C. The region shown is that of the COOH and NH protons

two signals are due to protons which belong to the same pyrromethenone unit. The δ 11.23 and 10.49 peaks were assigned to 21- and 22-H, respectively, by comparison with the spectrum of the model compound VBA methyl ester (see below) which is an analogue of the pyrromethenone unit in bilirubin dimethyl ester.

The methine and methyl peaks in the spectrum of the dimethyl ester were assigned on the basis of NOE measurements. An irradiation at δ 1.98 produced an NOE of 0.23 on the methine peak at δ 5.90. An irradiation at the signal of the two methyl groups at δ 2.08 produced NOEs of 0.18 and 0.22 on the methine peaks at δ 6.20 and 5.90, respectively. No NOE was observed when the methyl resonance at δ 1.75 was irradiated. Thus this resonance belongs to the 2-methyl group and one of the δ 2.08 peaks is due to the 7-methyl group. Considering the chemical similarity of the 7- and 13-methyl groups, it is reasonable to assign the other peak at δ 2.08 to the 13-methyl group, so that the peak at δ 1.97 must belong to the 17-methyl group. The NOEs above also show that the methine peaks at δ 6.20 and 5.90 belong to 5- and 15-H, respectively.

VBA methyl ester. A partial assignment of the spectrum has been published by Manitto and Monti.[20] In the present work, specific assignments of the NH signals were obtained by spin-decoupling experiments, where irradiation of the 9-H resonance at δ 6.87 caused a narrowing of the resonance of the pyrrole NH. The assignment of the NH signals agrees with that reported by Manitto and Monti.[20] The assignments of the

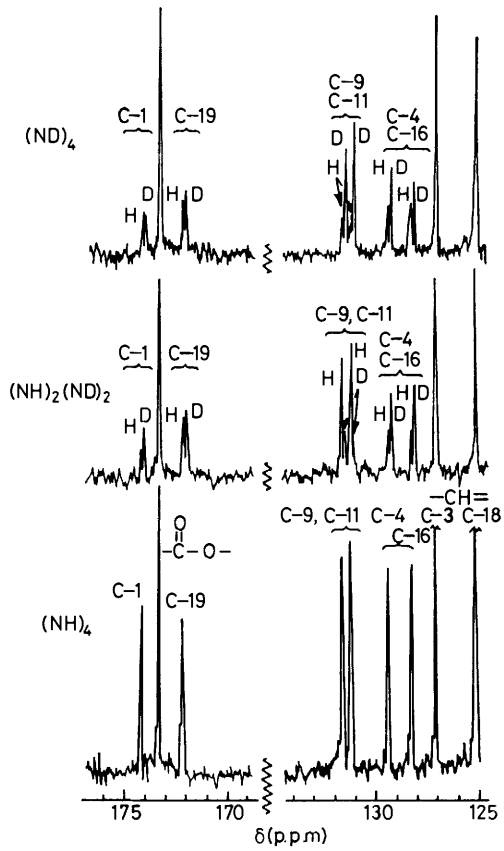

FIGURE 3 Portions of the ^{13}C spectra of bilirubin dimethyl ester [$(NH)_4$], the same compound with two of its NH protons replaced with deuterons [$(NH)_2(ND)_2$], and with all the four NH protons replaced with deuterons [$(ND)_4$]. The letters H and D in the spectra designate signals of carbons attached to NH and ND groups, respectively

signals of methyl protons were verified by NOE experiments where an NOE was produced on the peak of methine proton by irradiation at the signal of the 7-, but not of the 2-methyl group.

II *Nuclear Overhauser Effects and Spin–Lattice Relaxation Times.*—Qualitatively, the most significant results related to the conformation of bilirubin and its derivatives are the observed nuclear Overhauser enhancements (NOE). The observation of an NOE between the lactam and pyrrole NH protons (see, *e.g.* Figure 2) as well as between methyl and methine CH protons (Table 3) indicates a *syn-Z* structure. Moreover, the observation

TABLE 3

Spin–lattice relaxation times and nuclear Overhauser enhancements in mesobilirubin, bilirubin, and bilirubin dimethyl ester in $CDCl_3$ solutions [a]

		Relaxation times of observed protons							NOE on observed resonance	
		T_1^{ns}/s		T_1^{s}/s		$\Sigma\eta$ (from T_1^{s}/T_1^{ns}				
	Observed resonance	30 °C	−20 °C	30 °C	−20 °C	30 °C	−20 °C	Irradiated resonance	30 °C	−20 °C
Mesobilirubin [b]	22- and 23-H	0.43	0.186	0.54	0.23	0.26	0.24	10-CH_2 protons	0.05	nd
	22- and 23-H							21- and 24-H	0.23	0.22
	21- and 24-H	0.41	0.184	0.55	0.25	0.34	0.36	22- and 23-H	0.23	0.25
								COOH protons	0.06	0.06
	COOH protons		1.03 [c]					21- and 24-H	0.08	0.11
	10-CH_2 protons	0.278	0.128							
Bilirubin [d]	22- and 23-H	0.47						21-H	*f*	
	22- and 23-H							24-H	*f*	
	21-H	0.36 [e]						22- and 23-H	*f*	
	24-H	0.39 [e]						22- and 23-H	*f*	
	10-CH_2 protons	0.28								
Bilirubin [g] dimethyl ester	22-H	0.16		0.20		0.25		10-CH_2 protons	0.05	
	22-H							21-H	0.12	
	23-H	*h*						10-CH_2 protons	0.05	
	21-H	0.18		0.21		0.17		22-H	0.12	
	10-CH_2 protons	0.090						22-H	0.02	
	10-CH_2 protons							23-H	0.02	
	5-H	*h*						7-CH_3 protons	0.18	
	15-H							17-CH_3 protons	0.22	
	15-H	*h*						13-CH_3 protons	0.23	

[a] The results were obtained at a resonance frequency of 90 MHz; the estimated random errors were generally ±5% in the T_1 values and *ca.* ±10% in the NOE values, except for NOEs smaller than 0.08 where the random errors were *ca.* ±20%. [b] The concentration was 0.009M at a temperature of 30 °C and slightly lower at −20 °C. [c] Value obtained by the method of Mann (see text). [d] Values obtained on a 0.0015M solution at a temperature of 30 °C. [e] Values may be interchanged. [f] A positive NOE was observed but was not measured quantitatively. [g] Values obtained on a 0.17M solution at a temperature of 30 °C; the random errors of the T_1 values in this case were ±10—15%. [h] No T_1 measurements could be made for this proton because of an excessive overlap with other signals.

nd = Values not determined.

of an NOE between the COOH and lactam protons of mesobilirubin (Figure 2) provides a clue for the conformation of this compound in solution.

In order to calculate the interproton distances, the spin–lattice relaxation times (T_1), the nuclear Overhauser enhancements (NOE), and the rotational correlation times of the pairs of protons in question must be known. In the present work, the relaxation parameters were extracted from three types of experiments: (1) non-selective T_1 measurements (T_1^{ns}), where the magnetizations of all the protons are simultaneously excited; (2) selective T_1 measurements (T_1^{s}), where a long, weak pulse is used to excite exclusively the magnetization of the observed proton; and (3) steady-state NOE measurements, in which one determines the fractional enhancement of the signal of one proton produced by irradiating the signal of another proton which interacts dipolarly with it. Various aspects of the theory behind these experiments have been treated by several investigators.[11,17,22] The application of their results to the present situation is summarized below.

Consider a proton i which is being relaxed by intramolecular dipolar interactions and is involved in processes of chemical exchange. Denoting the value of the Z-component of the magnetization at a given moment by M_Z and its equilibrium value by M_0, the general equation of relaxation for the proton i is (1).[22] In equation (1), ρ_{ij} is the direct dipolar relaxation rate, σ_{ij} is the cross-relaxation rate, k_{ij} is the pseudo-first-order rate of exchange between i and j, and the summation is

$$dM_z^{(i)}/dt = -(\sum_{j\neq i}\rho_{ij} + \rho_i^*)(M_z^{(i)} - M_0^{(i)}) - \sum_{j\neq i}[\sigma_{ij}(M_z^{(j)} - M_0^{(j)}) - k_{ij}M_z^{(i)} + k_{ij}M_z^{(j)}] \quad (1)$$

carried over all the protons which have a dipolar interaction or exchange with i. The term ρ_i^* includes contributions to the relaxation from dipolar interactions with ^{14}N nuclei, deuterons on solvent molecules, or interactions with paramagnetic impurities.

In the absence of chemical exchange, the initial rates of relaxation in the non-selective and selective T_1 measurements, R^{ns} and R^{s}, are given by equations (2) and (3).[17] Since the NOE, $\eta_i(j)$ is given by $\eta_i(j) = \sigma_{ij}/(\sum_{j\neq i}\rho_{ij} + \rho_i^*)$, we have equation (4). Equation (4) thus yields the sum of all the possible NOEs on the resonance of i due to dipolar interactions with other protons. Each value of $\eta_i(j)$ can be obtained, in principle, by doing experiment 3 as described above. If the condition of extreme narrowing prevails, *i.e.* if $\omega\tau \ll 1$

$$R_i^{ns} = \sum_{j\neq i}(\rho_{ij} + \sigma_{ij}) + \rho_i^* \quad (2)$$

$$R_i^{s} = \sum_{j\neq i}\rho_{ij} + \rho_i^* \quad (3)$$

$$R_i^{ns}/R_i^{s} = 1 + \sum_{j\neq i}\eta_i(j) \quad (4)$$

where ω is the resonance frequency in rad s^{-1} and τ is the rotational correlation time modulating the dipolar interaction, we have $\sigma_{ij} = \rho_{ij}/2$. The average distance between protons i and j, r_{ij} is then related to the relaxation parameters by expression (5) where τ_{ij} is the rotational correlation time of the vector r_{ij}.

$$\langle r_{ij}^{6} \rangle = 0.5\gamma_{H}^{4}\hbar^{2}T_{1}^{s}\tau_{ij}/\eta_{i}(j) \qquad (5)$$

When an exchange process is operative and the rate of exchange is of the same order of magnitude as the spin–lattice relaxation time, the recovery of $M_z^{(i)}$ becomes dependent on the rate of exchange, especially in the selective T_1 experiment, and equation (3) must be modified.

The values of T_1^{ns}, T_1^{s}, and $\eta_i(j)$ for protons in mesobilirubin, bilirubin, and bilirubin dimethyl ester in $CDCl_3$ are shown in Table 3. In several instances given in Table 3, resonances of protons situated in symmetric positions in the two pyrromethenone units appear at identical chemical shifts, and for these resonances no separate T_1 and NOE values could be measured. However, the fact that the T_1 plots did not show any sign of deviation from exponentiality precluded the possibility of large differences (greater than a factor of two) between the T_1 values of the two protons contributing to the same resonance.

The relaxation parameters and their structural implications in the different compounds are discussed below.

Mesobilirubin. The relaxation of the lactam protons 21- and 24-H, and of the pyrrole protons 22- and 23-H was found to be unaffected by chemical exchange. In a saturation-transfer experiment, it was found that the intensities of the signals of the NH protons were not changed when the signal of the residual water which was present in the sample (0.025M) was saturated. In addition, the NOE produced on the signal of the lactam protons by irradiating the signal of the COOH protons was found to be independent of the temperature. Thus both intermolecular exchange with water and intramolecular exchange with the carboxylic acid protons are negligibly slow in comparison to the other relaxation mechanisms of the NH protons.

The relaxation of the NH protons in mesobilirubin is thus dominated by homonuclear dipolar interactions and dipolar interactions with ^{14}N nuclei. For all the NH protons, the values of the total contribution of the interactions with neighbour protons, $\Sigma\eta$, is considerably less than 0.5 (Table 3). Thus, heteronuclear dipolar interaction with ^{14}N contributes *ca.* 50% to the relaxation of these protons. For the pyrrole NH protons, 22- and 23-H, $\Sigma\eta$ is fully accounted for by the sum of NOEs produced by irradiations at the resonances of the lactam and the bridge CH_2 protons. For the lactam protons, the sum of NOEs from interactions with the pyrrole and COOH groups is slightly lower than $\Sigma\eta$, a result which may indicate that other protons in the molecule interact dipolarly with the lactam protons. However, in view of the large experimental errors in the values of $\Sigma\eta$, the above comparisons between the sum of measured NOEs and $\Sigma\eta$ are not regarded here as conclusive.

The NOE produced on the resonance of the COOH protons upon the irradiation of the lactam resonance (Table 3) shows that a dipolar interaction with the lactam protons is operative for the relaxation of the carboxylic acid protons. The irradiation of resonances from protons other than the lactams did not yield any NOE on the COOH resonance, so that the only protons in the molecule of mesobilirubin that have a dipolar interaction with the COOH groups are the lactam protons.

The small value of the NOE observed on the COOH resonance, in comparison with the theoretical value of 0.5, results from the presence of another relaxation mechanism, namely, the transfer of magnetization through proton exchange with residues of water in the solution. This exchange prevented the observation of NOE between these groups. The saturation of the H_2O resonance at room temperature caused a complete disappearance of the COOH resonance, indicating that the rate of exchange is rapid compared with the longitudinal relaxation rate. A dipolar interaction between the COOH and water protons is very unlikely. One reason to believe that there is no tight binding of the water molecules to mesobilirubin is the absence of a change in the chemical shift of the water resonance in the presence of mesobilirubin. Following equation (1), the equation of relaxation for the carboxylic acid protons in the presence of water is thus (6) where A signifies the

$$dM_z^{A}/dt = -R_A(M_z^{A} - M_0^{A}) - \sigma_{AL}(M_z^{L} - M_0^{L}) - \sigma_{AW}(M_z^{W} - M_0^{W}) - k_{AW}M_z^{A} + k_{WA}M_z^{W} \qquad (6)$$

carboxylic acid protons and L and W are the lactam and water protons, respectively. We shall assume that ρ^* is negligible for the carboxy-protons.

In our NOE experiments, the lactam resonance is saturated, *i.e.* $M_z^{L} = 0$, and $dM_z^{A}/dt = 0$ by the steady-state condition. From equation (6), we have (7) where

$$1 + \eta_A(L) = [\rho_{AL} + k_{AW}(M_z^{W}/M_0^{W}) + \sigma_{AL}]/(\rho_{AL} + k_{AW}) \qquad (7)$$

the equilibrium conditions $M_0^{L} = M_0^{A}$ and $k_{AW}M_0^{A} = k_{WA}M_0^{W}$ were used. The ratio M_z^{W}/M_0^{W} was found to be greater than unity in the above NOE experiment. This effect does not result from a dipolar interaction between the lactam and water protons, since no NOE was found when the water signal was saturated. The reason of M_z^{W}/M_0^{W} being greater than unity is a secondary NOE, which is caused by the NOE on the COOH resonance and the saturation transfer to the COOH protons *via* exchange.

Equation (7) contains three unknowns, ρ_{AL}, σ_{AL}, and k_{AW}. Fortunately, the proton exchange between the carboxylic acid and water at −20 °C was sufficiently slow, and the separation between the two signals sufficiently large (*ca.* 1 100 Hz) for an independent measurement of k_{AW} and of $\rho_{AL} + \sigma_{AL}$ by the method of Mann.[23] These measurements yielded $k_{AW} = 5.1\ s^{-1}$ and $1/T_{1A}^{ns} = \rho_{AL} + \sigma_{AL} = 0.97\ s^{-1}$.

In the NOE experiment, $\eta_A(L)$ and M_z^W/M_0^W were found to be 0.11 ± 0.01 and 1.075 ± 0.010, respectively. By inserting the above value into equation (7), one obtains $\rho_{AL} = 0.71\ s^{-1}$ and $\sigma_{AL} = 0.26\ s^{-1}$, which leads to $\sigma_{AL}/\rho_{AL} = 0.37$. This experimental value is smaller than the value of 0.5 which is predicted by the theory. We note, however, that in contrast to k_{AW} and T_{1A}^{ns}, which were determined experimentally within a small random error, the values of ρ_{AL} and σ_{AL} as calculated from equation (7) may be subject to large propagated errors, and their present values are hence considered to be estimates.

Bilirubin. The T_1^{ns} values of the NH and bridge CH_2 protons (Table 3) are similar to the corresponding values in mesobilirubin. The appearance of NOEs on the lactam resonances upon the irradiation of the pyrrole NH resonances and *vice versa* indicates that a dipolar interaction between each lactam and its neighbour pyrrole NH contributes to the relaxation of both protons. Unfortunately, the very low solubility of bilirubin in chloroform made further NOE measurements impractical.

Bilirubin dimethyl ester. Interpretation of the relaxation parameters in this case is complicated by the occurrence of the compound as a dimer at a concentration of *ca.* 0.016M.[9,10] If a further aggregation were occurring at high concentrations, the relaxation times would be expected to depend on the concentration.

The spin–lattice relaxation times of the protons of bilirubin dimethyl ester were measured as a function of the concentration. The value of T_1^{ns} of the bridge CH_2 protons and the NH protons in a 0.01M solution were found to be longer only by 10% than their values at a concentration of 0.17M. The slight dependence of the T_1^{ns} values on the concentration is believed to reflect the dependence of the correlation times on the viscosity of the solution rather than formation of higher aggregates. It may thus be concluded that in the range of concentrations from 0.01 to 0.17M, bilirubin dimethyl ester occurs as a dimer in chloroform solutions.

A comparison between the results of bilirubin dimethyl ester and those of bilirubin and mesobilirubin (Table 3) shows that the effect of dimerization may be more than a simple change of the correlation time. The T_1 values for the ester are shorter by a factor of 2.5—3, rather than 2.0, than those for bilirubin and mesobilirubin. No transfer of saturation was observed between the NH protons and water, indicating that the relaxation of the protons is not affected by exchange. For the lactam proton, 21-H, and the pyrrole proton, 22-H, the sums of the measured NOEs are smaller than the corresponding values in mesobilirubin. This effect, and the shortening of the T_1 values described above, may both be attributed to intermolecular dipolar relaxation within the dimer. However, our present results are not sufficient for a quantitative determination of these interactions.

III *Interproton distances in mesobilirubin and in bilirubin dimethyl ester.* In order to calculate interproton distances from equation (5), the values of the correlation times for reorientation of the interproton vectors, τ_{ij}, are required. In our previous ^{13}C study of bilirubin and some of its derivatives,[9] the reorientation of the backbone of these molecules was found to be determined by a single isotropic correlation time, τ_R. Following this result, the calculation of distances was simplified in the present work by assuming, for all the protons involved, that τ_{ij} is approximately equal to τ_R. However, the solubility of mesobilirubin in chloroform was found to be insufficient for ^{13}C n.m.r. measurements. The value of τ_R of mesobilirubin was estimated on the basis of τ_R of bilirubin in dimethyl sulphoxide (DMSO), assuming (a) that bilirubin and mesobilirubin have the same correlation time in chloroform, and (b) that the ratio of the τ_R values of bilirubin in DMSO and chloroform is determined by the ratio of the viscosities of the solution. These assumptions are based on the following considerations. The relaxation of the bridge CH_2 protons attached to C-10 depends mostly, though not exclusively, on their mutual interaction, and should hence be insensitive to the specific conformation of the molecule. The relaxation time of these protons is thus expected to be inversely proportional to the viscosity of the solution. This is indeed the case in bilirubin, as the ratio of the T_1 values of the bridge CH_2 protons in $CDCl_3$ (Table 3) and $[^2H_6]$DMSO [9] is 0.29, which is close to the ratio of 0.25 between the viscosities of a 0.05M solution of bilirubin in $[^2H_6]$DMSO and of chloroform. From these results, and from the correlation time of 2.2×10^{-10} s of bilirubin in DMSO, the value of τ_R of mesobilirubin in $CDCl_3$ was

TABLE 4

Interproton distances (Å) in mesobilirubin, bilirubin, and bilirubin dimethyl ester, as calculated from the n.m.r. relaxation data (Table 3) and from the *X*-ray data (refs. 7 and 8)

				Distances between protons					
	Method	Temperature (°C)	$10^{10}\tau_R$/s	22-H–10-H(2)	23-H–10-H(1)	21-H–22-H	23-H–24-H	12-COOH–21-H	8-COOH–24-H
Mesobilirubin	N.m.r.	30	0.59 [a]	2.4		1.9		2.3	
		−20	1.4 [a]			1.9		2.3	
	X-Ray			2.86	2.45	1.75	2.08	2.6 [b]	2.3 [b]
Bilirubin	*X*-Ray			2.79	2.84	1.54	1.58	2.53	2.45
Bilirubin dimethyl ester	N.m.r.	30	1.7 [c]	2.4		2.1			

[a] Estimated from the τ_R value of bilirubin in $[^2H_6]$DMSO solutions (see text). [b] Estimated from the positions of the carbon and oxygen atoms of the carboxylic acid groups and lactam NH groups reported in ref. 8. [c] Value obtained from ^{13}C relaxation data (ref. 9).

estimated to be 0.59×10^{-10} s at 30 °C. The temperature-dependence of the T_1 value of the bridge CH_2 protons of mesobilirubin (Table 3) is approximately as predicted by the corresponding change of viscosity. Since the accurate viscosity of chloroform at −20 °C was not available, τ_R at this temperature was estimated to be *ca.* 1.4×10^{-10} s on the basis of the temperature dependence of T_1 of the bridge CH_2 protons alone.

The interproton distances obtained for mesobilirubin in $CDCl_3$ solutions are given in Table 4.

Consider first the distances between the pyrrole NH and the bridge CH_2 protons. The relaxation of each of the pyrrole NH protons is a function of its interactions with both of the bridge CH_2 protons. These interactions depend on the orientations of the pyrrole rings B and C relative to the bridge CH_2 group, which may be defined through the dihedral angles τ_1 and τ_2 subtended by 10-H(2), C-10, C-9, and N-22 and by 10-H(1), C-10, C-11, and N-23, respectively. By using the atomic coordinates of the pyrrole NH and bridge CH_2 protons reported for the crystal structure of mesobilirubin,[8] we calculated the dipolar interaction terms, r_1^{-6} and r_2^{-6}, corresponding to the interactions of each pyrrole NH with 10-H(1) and 10-(2), as a function of the dihedral angles τ_1 and τ_2. The dependence of the terms r_1^{-6}, r_2^{-6}, and their sum $r_1^{-6} + r_2^{-6}$ on the orientation of the pyrrole rings is depicted in Figure 4.

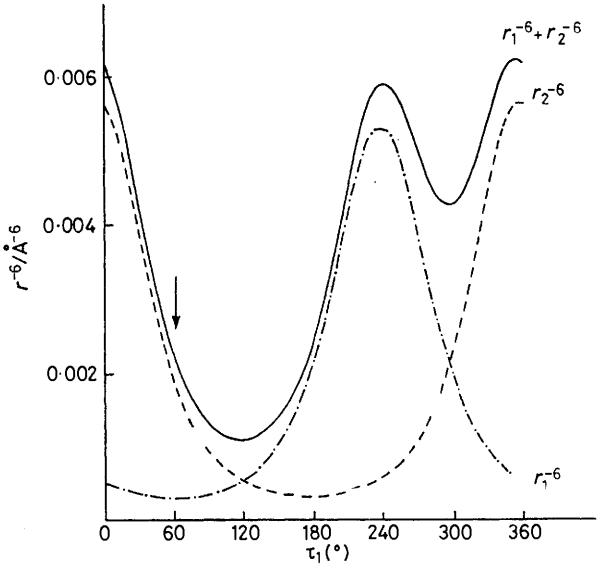

FIGURE 4 Dipolar interactions between the pyrrole proton 22-H and the two bridge protons on C-10 of mesobilirubin, expressed in reciprocal sixth power distances as a function of the dihedral angle. The arrow indicates the values of τ, in the crystal.

The distances between each lactam proton and its neighbouring pyrrole NH proton in mesobilirubin indicate that the structure about the methine bonds is *syn-Z*. The distance of 2.3 Å between each lactam and its neighbouring COOH proton is evidence of hydrogen bonding between the carboxy-proton and the oxygen atom of the ring carbonyl. The similar values of the lactam–pyrrole NH distances at temperatures of −20 and 30 °C, and the existence of the NOE between the lactam and carboxy-protons at 30 °C, indicate that the conformation is practically unchanged at this range of temperatures.

Theconformation of mesobilirubin which conforms to the interproton distances and the hydrogen bonding is shown in Figure 5.

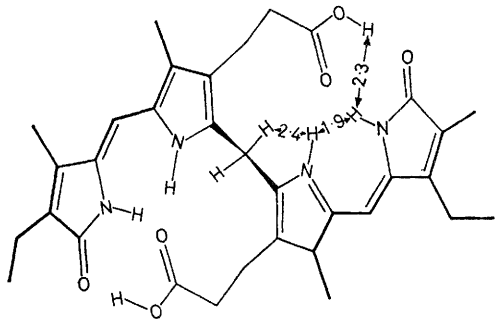

FIGURE 5 Conformation and interproton distances (Å) for mesobilirubin in chloroform solutions, obtained from the n.m.r. relaxation data

It may be useful to compare the interproton distances in solution as calculated from the n.m.r. data and those reported or estimated from the *X*-ray studies of bilirubin and mesobilirubin (Table 4). The distances between the lactam and pyrrole NH protons and between the lactam and COOH protons are similar in solution and in the crystal. The distance between each of the bridge CH_2 proton and its nearest pyrrole NH neighbour in solution is appreciably smaller than in the crystal. However, the error in the estimation of these distances by n.m.r., and the error in their estimation by the *X*-ray method, where the protons are not observed directly, do not allow a conclusion concerning the difference between the conformations in the crystal and solution. The lactam–pyrrole NH distances in bilirubin, obtained in the *X*-ray study by an indirect measurement, are unreasonably short. These values are entirely different from our n.m.r. distances and also from the corresponding values in crystalline mesobilirubin.

Some interproton distances were calculated for bilirubin dimethyl ester in chloroform solutions, by assuming that the relaxation is determined exclusively by intramolecular interactions, and using the value of 1.7×10^{-10} s for the correlation time of the dimer.[9] The distance between the lactam proton 21-H and the pyrrole proton 22-H could be calculated using either the T_1^s and NOE values for 21-H or the corresponding values for 22-H. In fact, the two calculations yielded the same value of 2.1 Å. The interproton distances in bilirubin dimethyl ester are very similar to the corresponding values in mesobilirubin in chloroform solutions, confirming our previous conclusion, namely that any intermolecular dipolar interactions that may be present within the dimer of bilirubin dimethyl ester in chloroform contribute little to the relaxation.

IV *Exchange of* NH *Protons with Methanol.*—The rates of exchange of the pyrrole NH protons of VBA methyl

ester, mesobilirubin, bilirubin, and bilirubin dimethyl ester with deuteriated methanol are given in Table 5. The exchange is fastest for VBA methyl ester, indicating

TABLE 5

Rates of proton exchange (s^{-1}) of lactam and pyrrole NH groups of bilirubin and its derivatives with methanol

	Pyrrole NH [a]	Lactam [b,c]			
[methanol]/M	0.3	0.025	0.125	0.25	0.5
VBA methyl ester	1×10^{-2}	0.3	2.3	6	10
Mesobilirubin	$<4 \times 10^{-5}$	0.24	1.7	2.4	7
Bilirubin	$<4 \times 10^{-5}$				
Bilirubin dimethyl ester	4×10^{-3} (22-H) 3×10^{-3} (23-H)	<0.2 [d]	0.45 [d]	1.1 [d]	2.5 [d]

[a] Deuterium exchange with CD_3OD, determined from the time dependence of the intensity of the NH resonance. The measurements were done at a resonance frequency of 300 MHz at *ca.* 23 °C. The concentrations were *ca.* 0.04M for VBA methyl ester and bilirubin dimethyl ester, 0.01M for mesobilirubin, and *ca.* 0.0017M for bilirubin. [b] Proton exchange with CH_3OH, measured by the method of Mann.[23] The resonance frequency was 90 MHz and the temperature 30.0 °C. The measurements were done on 0.009M solutions of the compounds in dry $CDCl_3$. [c] The random errors of the exchange rates, caused mainly by a low signal-to-noise ratio, are *ca.* ±10—30%. [d] Exchange rates 21-H.

that in this compound the pyrrole NH protons are most accessible to the methanol molecules. The slow exchange of the pyrrole NH protons of bilirubin dimethyl ester is reasonable, as these protons may be ' buried ' within the dimer. In comparison with these two compounds, the exchange of the pyrrole NH protons in mesobilirubin and bilirubin is extremely slow. This result indicates that in mesobilirubin and bilirubin these protons are sterically shielded from the methanol molecules, most probably because of hydrogen bonding between these protons and the carbonyl oxygens of the carboxy-groups.

In all the compounds studied in this work, the rates of exchange of the lactam protons with methanol are much faster than those of the pyrrole NH protons and are comparable to their spin–lattice relaxation times. These exchange rates could thus be measured by the saturation–transfer technique. The results for VBA methyl ester, mesobilirubin and bilirubin dimethyl ester at a fixed concentration of the pigment and different concentrations of methanol are also given in Table 5.

For each compound, the rate of exchange is proportional to the concentration of methanol within experimental error. The exchange of the lactam protons of bilirubin dimethyl ester is slower than that of VBA methyl ester by a factor of about three, which reflects, as observed for the pyrrole NH protons, steric hindrance about the lactam group within the dimer of bilirubin dimethyl ester. In mesobilirubin, however, the exchange of protons between the lactam groups and methanol is only slightly slower than in VBA methyl ester. This result is surprising because the lactam protons of mesobilirubin in $CDCl_3$ solutions are hydrogen-bonded and any interaction with the methanol molecules should be sterically hindered. It is quite possible, however, that the lactam group of VBA methyl ester is not freely accessible to the methanol. In recent studies by Falk *et al.*,[24] several pyrromethenone compounds were found to dimerize in chloroform. If a similar dimerization occurs for VBA methyl ester, it probably involves intermolecular hydrogen bonding through the lactam groups, leading to slow exchange rates of the lactam NH protons. Alternatively, the relative rapidity of the exchange of the lactam protons of mesobilirubin with methanol may be due to a catalytic effect of the COOH groups through the formation of a pseudolactim structure. Such a structure may increase the acidity of the lactam protons and thus compensate, in part, for the retardation of their exchange rates by the steric hindrance. Since a similar mechanism does not apply to the pyrrole NH protons, their exchange with methanol is only affected by the steric hindrance and is thus very slow.

V *Conclusions.*—The interproton distances as obtained for the molecule of mesobilirubin show unequivocally that its conformation in chloroform solutions involves internal hydrogen-bonding of the COOH protons to the oxygen atoms at C-1 and C-19 of the pyrrolenone rings. The very slow exchange rate of the pyrrole NH protons with methanol indicates that these groups are hydrogen-bonded to the carboxy-groups. In view of the conclusions above, it is very probable that the lactam protons are also hydrogen-bonded to the carboxylic groups.

The ^1H n.m.r. spectrum of bilirubin, especially the chemical shifts of the NH and COOH protons which are sensitive to the conformation, are very similar to the corresponding chemical shifts in mesobilirubin. The rate of deuteriation of the pyrrole NH groups is similar to that of mesobilirubin. The relaxation times of the NH protons and the bridge methylene protons are similar in the two compounds. The above mentioned results constitute evidence that the conformation of bilirubin and mesobilirubin in chloroform solutions are similar. Thus, the present study confirms the suggestion of Kuenzle *et al.*[4] with respect to the structure of bilirubin in solution.

In a recent study, Holzwarth *et al.*[10] have raised the possibility that bilirubin dimethyl ester may be present as a mixture of *syn-* and *anti*-isomers in various solvents. The results of our NOE measurements concerning this compound indicate that the configuration about both methine bonds is *syn-Z* in chloroform solutions, as it is in bilirubin and its other derivatives discussed in the present work.

The properties of bilirubin and its dimethyl ester are very different in chloroform solutions. The ester is readily soluble, forms dimers over a wide range of concentration and its propionic side-chains, as shown by their ^{13}C relaxation times,[9] are not hydrogen-bonded. The exchange of protons between the pyrrole NH groups and methanol is much faster in the ester than it is in bilirubin and mesobilirubin. It is noteworthy that intermolecular dipolar interactions within the dimer of bilirubin dimethyl ester play only a minor role in the relaxation of the NH protons of the molecule.

Preliminary measurements have indicated to us that in DMSO solutions, the conformations of both bilirubin and its dimethyl ester are significantly different from their conformations in chloroform solutions. This subject will be described in a later publication.

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