A Nuclear Magnetic Resonance Study of the Conformation and the Interconversion between the Enantiomeric Conformers of Bilirubin and Mesobilirubin in Solution

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^{1}H N.m.r. spectra of bilirubin and mesobilirubin in chloroform solutions and their temperature dependence were studied. At room temperature the spectrum of the methylene protons of the two propionic acid residues appear as two partially overlapping ABCX multiplets. Specific assignments of the signals were obtained through the ^{1}H–^{1}H nuclear Overhauser enhancement and selectively proton-decoupled ^{13}C spectra. Approximate conformation of the propionic acid residues in terms of the dihedral angles within the CH_2CH_2 residue and its angles relative to the rest of the molecule was determined on the basis of the chemical shifts, geminal, and vicinal coupling constants. The results are consistent with previous determinations of the conformation of bilirubin and mesobilirubin in chloroform solutions and in the solid state. At elevated temperatures the spectral lineshape of the propionic methylene protons of bilirubin and mesobilirubin changes drastically. The changes were successfully simulated using the DNMR5 computer program, assuming an exchange of the type ABCX A′B′C′X′ $\rightleftharpoons$ XCBA X′C′B′A′, caused by inversion between the two enantiomeric conformations of these molecules. The activation parameters of the exchange are $\Delta H^{\ddagger}$ 74.1 ± 2.5 kJ mol^{-1} and $\Delta S^{\ddagger}$ −8.8 ± 7.1 J K^{-1} mol^{-1} for bilirubin and $\Delta H^{\ddagger}$ 72.4 ± 1.2 kJ mol^{-1} and $\Delta S^{\ddagger}$ − 12.5 ± 3.8 J K^{-1} mol^{-1} for mesobilirubin.

Bilirubin is a natural bile pigment formed mainly in the catabolism of heme. Abnormally high levels of bilirubin in the blood may lead to brain damage, especially in jaundiced newborn children.[1] Previous studies of proton relaxation, nuclear Overhauser enhancement (NOE), and exchange rates[2] have established that the conformation of bilirubin in chloroform solutions is that shown in Figure 1 [(I)], as originally proposed by Kuenzle and his co-workers.[3]

The interconversion between conformation (I) and its mirror image (II) (Figure 1) proceeds *via* rupture of the six hydrogen bonds followed by rotation of each of the pyrromethenone fragments about the central CH_2 bridge (C_{10}) and re-formation of the hydrogen bonds. An n.m.r. study of this process in modified bilirubin, where an asymmetric centre was introduced by converting one of the vinyl groups into $CH(CH_3)SCOCH_3$, was reported by Manitto and Monti.[4] However, only one rate constant at the coalescence temperature was estimated.

Recent 300 MHz n.m.r. studies have revealed that the fixed orientation of the propionic residues in conformation (I) is clearly manifested by the spectrum, since it removes the chemical equivalence of the two α- and two β-CH_2 protons.[5] Thus, the multiplets due to the propionic methylene protons appear as non-symmetric ABCX rather than the A_2B_2 pattern expected for rapidly rotating CH_2CH_2 moieties. Moreover, it can be seen from Figure 1 that the (I) $\rightleftharpoons$ (II) interconversion should involve, for the propionic CH_2CH_2 groups, a mutual exchange of the two non-equivalent α-protons and an analogous exchange of the β-protons.

In the present paper we report the assignment of the ^{1}H n.m.r. spectrum of the propionic residues of bilirubin and mesobilirubin, and its utilization for studying their conformation and interconversion in solution.

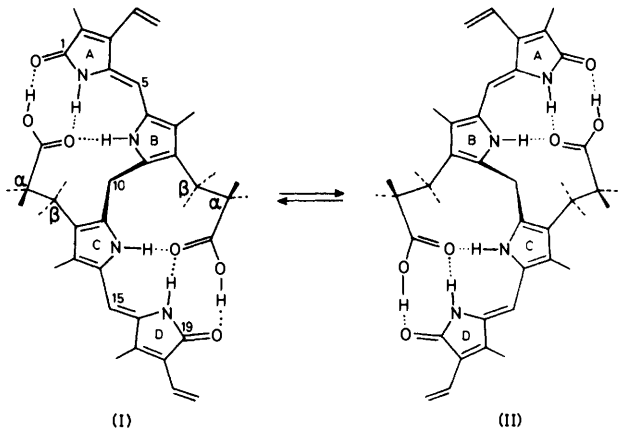

Figure 1. Exchange between the two enantiomeric hydrogen bonded conformers of bilirubin

Experimental

Materials.—Bilirubin was purchased from Sigma. Mesobilirubin was prepared by catalytic hydrogenation of bilirubin, according to procedures given in the literature.[6] Deuteriochloroform (99%D) was a Merck product.

Apparatus.—The n.m.r. measurements were done on a Bruker WH-90 NMR at Tel-Aviv University, a Bruker WH-300 at the Hebrew University in Jerusalem, a Bruker WH-270 at the Weizmann Institute of Science in Rehovot, and a Bruker WM-400 spectrometer at Bruker Analytische Messtechnik GMBH, Silberstreifen, West Germany.

Methods.—Saturated solutions of the compounds, that were *ca.* 0.0017 and 0.01M in bilirubin and mesobilirubin, respectively, at room temperature, were used. NOE were measured by difference spectroscopy as described previously.[2] ^{13}C Spectra of mesobilirubin were obtained at 75.5 MHz and required *ca.* 25 000 scans per spectrum. For the variable temperature ^{1}H spectra, samples were prepared in thick-wall 5 mm n.m.r. tubes (Wilmard Cat. No. 504-PP). Dissolved oxygen was removed by three freeze–pump–thaw cycles and the tubes were then sealed in flame. Sample temperatures were determined from the temperature dependencies of the chemical shifts of the pyrrole and lactam NH protons relative to that of

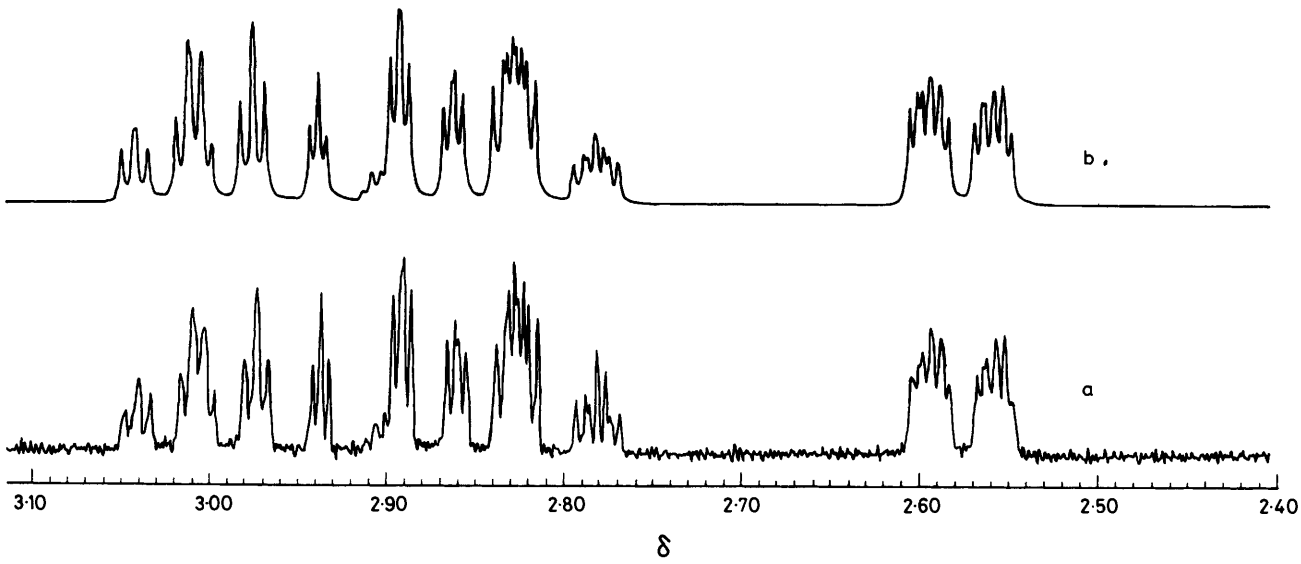

Figure 2. Portion of 400 MHz, resolution-enhanced ^1H n.m.r. spectrum of a saturated solution of bilirubin in chloroform: (a), number of accumulations was 2 000; (b) eight spin simulated spectrum, obtained by using the PANIC simulation program of the Bruker ASPECT 2000 computer

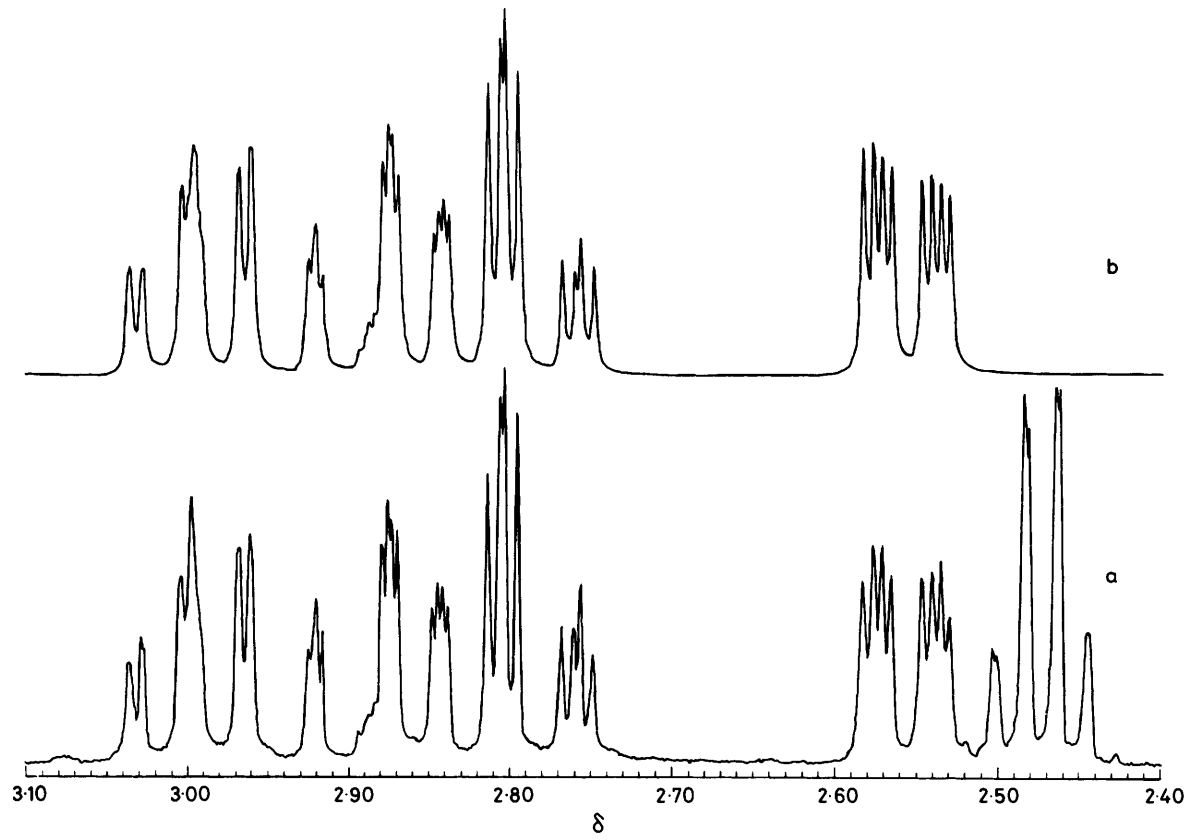

Figure 3. Experimental (a) and simulated (b) partial spectra of mesobilirubin. For details see caption of Figure 2. Number of scans in the experimental spectrum 500

$CHCl_3$ from the solvent. The temperature dependencies of these chemical shifts were calibrated from similar variable-temperature spectra that were run on the WH-90 spectrometer, where sample temperatures were determined with a copper–constantan thermocouple.

Results and Discussion

(I) *Assignment of the* ^1H *N.m.r. Signals of the Propionic Methylene Protons.*—Resolution enhanced ^1H n.m.r. superfluous spectra of the propionic CH_2CH_2 groups of bilirubin and mesobilirubin at room temperature are shown in Figures 2a

Table. Spectral parameters of the propionic methylene protons of bilirubin and mesobilirubin in $CDCl_3$ solutions

	Bilirubin		Mesobilirubin	
	δ	$10^{-3}\Delta\delta_{8'-12'}$ (p.p.m.)[a]	δ	$10^{-3}\Delta\delta_{8'-12'}$ (p.p.m.)[a]
A	3.00	−6.5	2.99	−1.7
B	2.89	−4.2	2.87	−3.5
C	2.81	5.2	2.79	0
X	2.57	4.0	2.55	0.7
	J/Hz		*J*/Hz	
AB	12.9		13.4	
AC	2.8		2.8	
AX	−14.9		−14.9	
BC	−18.8		−18.8	
BX	2.6		2.6	
CX	4.7		4.7	

[a] Differences between the chemical shifts of protons in the 8′-propionic residue and their counterparts in the 12′-residue [$\delta_A(8') - \delta_A(12')$, *etc.*].

and 3a. The spectra comprise two partially overlapping ABCX multiplets which are due to the two non-equivalent 8′- and 12′-propionic acid residues. Computer simulations of the spectra are given in Figures 2b and 3b.

Differentiation between α- and β-protons was achieved by off-resonance proton decoupling experiments on the ^{13}C n.m.r. signals of C_α and C_β in mesobilirubin, the positions of which were found to be δ 32.5 and 18.4 p.p.m., respectively. Irradiation at δ_A caused a near-complete decoupling of C_α, whereas the C_β signal appeared as doublet, with an apparent J_{CH} of 15 Hz. This result indicates that H_X is attached to C_β, and suggests that H_A is the other proton. Irradiation at $(\delta_B + \delta_C)/2$ decoupled C_α completely, thus confirming that H_B and H_C are attached to this carbon.

In the 300 MHz ^1H spectra of bilirubin and mesobilirubin, irradiation of the signal of 10-H_1,H_2 (δ 4.07)[7] produced NOE of 4% on the signals of H_A. Irradiation at δ_A produced NOE of 5% on the signal of 10-H_1,H_2 as well as NOE of 22% on the signals of H_X. Thus, H_A is the propionic methylene proton which is nearest to the bridge CH_2 group (see Figure 1).

(II) *Structural Information from the Spectral Parameters of the Propionic Methylene Protons.—Chemical shifts.* The chemical shifts of the propionic methylene protons of bile pigments are typically δ 2.5—2.7 for the α- and δ 2.8—2.9 for the β-protons.[7-10] In bilirubin and mesobilirubin, δ_X is shifted upfield by *ca.* 0.5 p.p.m. (Table), probably due to the aromatic ring currents, and hence indicates that H_X is situated above the plane of the ring.

Geminal HH *coupling constants.* According to the general correlation between J_{gem} and the plane between the CH_2 group and the adjacent π orbital,[11] the value of J_{AX} (Table) corresponds to an orientation of the β-CH_2 group such that one proton lies very near the plane of the pyrrole ring and the other lies out of plane. It thus supports the conclusion from the chemical shift data above. The value of J_{BC} corresponds to an angle of 20—40° between the α-CH_2 group and the π-orbital of the carboxy C=O group.

Vicinal HH *coupling constants.* The dihedral H–C–C–H angles within the CH_2CH_2 residues (φ in Figure 4) were determined from J_{vic} (Table 1) and the generalized Karplus equation proposed by Haasnoot *et al.*[12] They were found to be φ_{AC} 61, φ_{CX} 52, φ_{XB} 62, and φ_{BA} 165 or (equally possible) 195°.

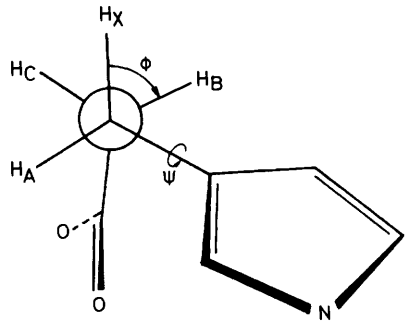

Figure 4. Conformation of the propionic acid residues in bilirubin and mesobilirubin

Similar values were obtained for mesobilirubin. Internal consistency requires that the sum $\varphi_{AC} + \varphi_{CX} + \varphi_{XB}$ be equal to the independently obtained value of φ_{BA}. Thus any deviation between these values reflects the error in the n.m.r. derivation of the angles. In view of the fact that the Karplus equation is based on force field calculations rather than experimental structural data, and the expected spread of these parameters for various molecules, the agreement here between $\varphi_{AC} + \varphi_{CX} + \varphi_{XB}$ and φ_{BA} seems satisfactory.

The structural information provided by the n.m.r. parameters above is qualitatively depicted in Figure 4. The similarity between bilirubin and mesobilirubin concerning their spectral parameters and structural information derived therefrom corroborates the previously indicated similarity[2] between their conformations. The values of the angles estimated here do conform with a structure of bilirubin and mesobilirubin in which the carboxy-groups are in the vicinity of the lactam NH groups.

Comparison with X-*ray data.* The detailed orientation of the propionic residues of bilirubin in the crystalline state was evaluated in terms of the dihedral angles (Figure 4) from the atomic parameters reported by Le Bas *et al.*[13] Some typical results are: ψ 8.6 and 123.1 for H_A and H_X, respectively, φ_{AC} 69, φ_{CX} 47, φ_{XB} 72, and φ_{BA} 188, and the torsional angle between C_α–H_B and the carboxy C=O group, 132°. Qualitatively, these results are in agreement with the structural features provided by the n.m.r. data, which conforms with the similarity between their crystalline state and solution conformations.

(III) *The Rate of the Interconversion between the Enantiomers of Bilirubin and Mesobilirubin.*—The n.m.r. spectrum of the propionic CH_2CH_2 residues undergoes a drastic change upon heating. As it can be seen in Figure 5, the well structured ABCX spectrum collapses to form a very broad spectrum which consists mainly of a broad signal with two very broad 'wings'. Qualitatively, this indicates an exchange between protons B and C which is close to the coalescence point, and an exchange between protons A and X which are more widely separated.

The system of methylene protons of the two propionic chains can be described as ABCX A′B′C′X′ $\rightleftharpoons$ XCBA X′C′B′A′. Attempts to apply the DNMR5 computer program of Binsch and Stephenson[14] to eight exchanging spins in a direct manner were unsuccessful owing to limitations on the processing time and memory size of our CDC 6600 computer. Instead, the problem was separated into two subsets, each consisting of four exchanging spins. The spectra of the subsets were calculated and then superimposed together to yield the calculated spectrum of the complete spin system. An estimate of the error in the fitted parameters was obtained by continuously varying them until a clearly visible difference between the simulated and experimental spectra could be observed.

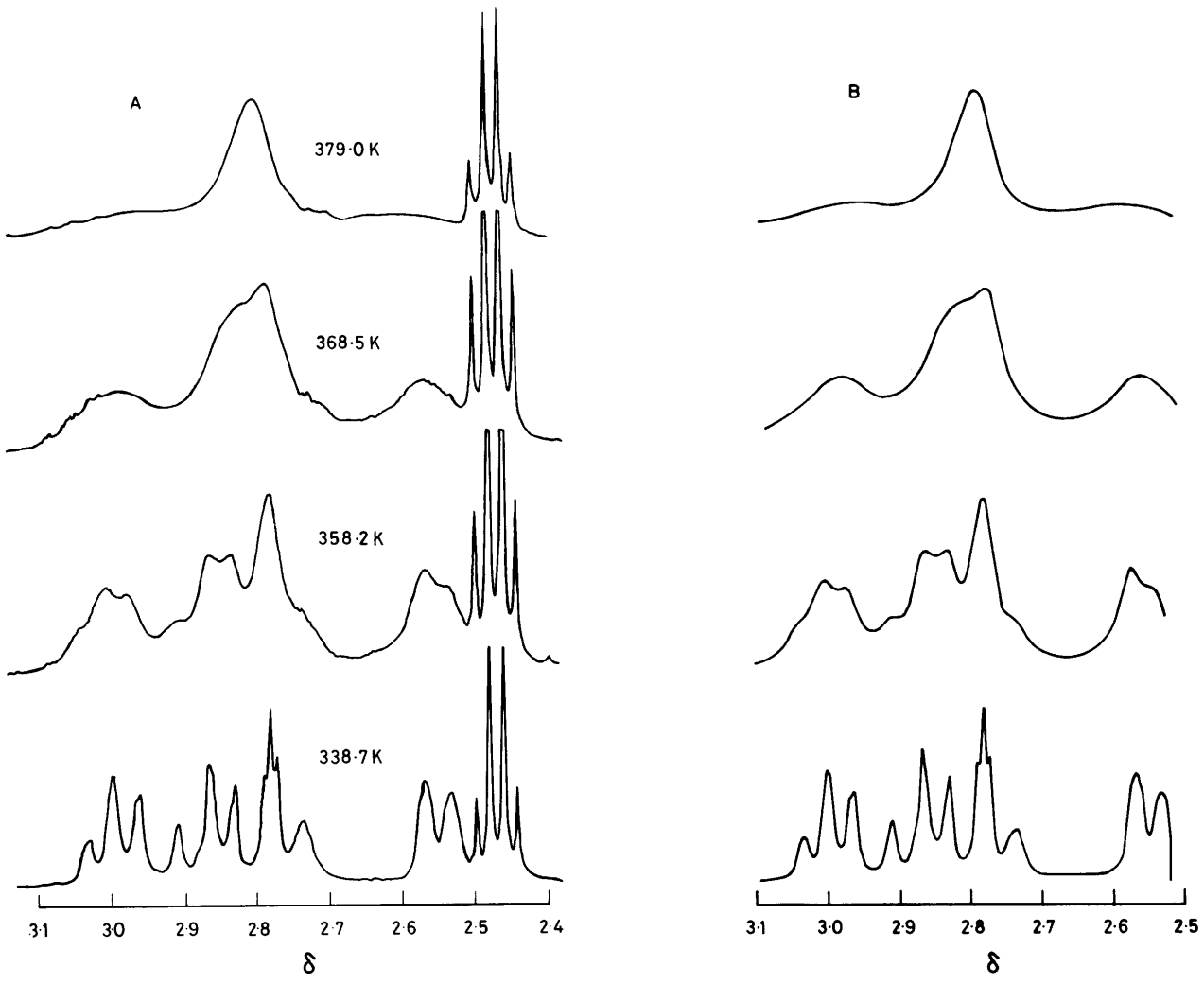

Figure 5. A, Typical spectra of the propionic methylene groups of mesobilirubin at elevated temperatures. Number of scans 100—300. B, Spectra simulated using the DNMR 5 computer program with exchange rate constants of 10.7, 44, 95, and 200 s^{-1} for the lower to the upper spectrum respectively

The simulated spectra of mesobilirubin at several temperatures are shown in Figure 5. Both experimental and simulated spectra of bilirubin were very similar to those of mesobilirubin. The temperature dependencies of the chemical shifts and Arrhenius plots for the rate constants, obtained from the computer simulation, are shown in Figures 6 and 7. The Arrhenius plots give, for bilirubin, $\Delta H^{\ddagger}$ 74.1 ± 2.5 kJ mol^{-1}, $\Delta S^{\ddagger}$ −8.8 ± 7.1 J K^{-1} mol^{-1}, and for mesobilirubin $\Delta H^{\ddagger}$ 72.4 ± 1.2 kJ mol^{-1}, $\Delta S^{\ddagger}$ −12.5 ± 3.8 J K^{-1} mol^{-1}. The value of $\Delta H^{\ddagger}$ 72—74 kJ mol^{-1} is consistent with the occurrence of six internal hydrogen bonds as depicted in Figure 1. The small value of $\Delta S^{\ddagger}$ is also reasonable since changes of entropy in such reactions are usually small.

Manitto and Monti[4] obtained a rate constant of *ca.* 7.2 s^{-1} at 53 °C for the interconversion between the diastereoisomers of devinyl-18-(1-methyl-2-acetylthio)bilirubin. The above value corresponds to a free energy of activation of 75 kJ mol^{-1} at 53 °C. As can be seen from Figure 7 the *k* values obtained in the present study at this temperature are 3.1 and 3.9 s^{-1} for bilirubin and mesobilirubin, respectively. These values are different by about a factor of two from the value reported for the modified bilirubin.[4] This result is somewhat unexpected as mesobilirubin, which is obtained from bilirubin by modifying both vinyl groups, behaves very similarly to bilirubin. However, since the result for the modified bilirubin[4] was determined from the coalescence point, its error should be evaluated before the effect of the modification on the rate constants is discussed.

Our experimental *k* values are overall rate constants, and do not indicate the details of the conformational interconversions, which probably involve partially hydrogen-bonded intermediates. A mere loosening of one propionic acid residue from its hydrogen bonds followed by restoration of the same conformation, *e.g.* conformation (**I**) in Figure 1 should have no effect on the n.m.r. spectrum. Only the full (**I**) $\rightleftharpoons$ (**II**) exchange brings about the coalescence. However, there are indications that the population of partially hydrogen-bonded intermediates is negligibly small even at high temperatures. Conformers with mobile, non-hydrogen bonded propionic residues are expected to have an A_2B_2 pattern as they do, *e.g.* in bilirubin dimethyl ester.[6] This means that the chemical shift differences $\delta_A - \delta_X$ and $\delta_B - \delta_C$ should go down to zero in such conformations. As it can be seen in Figure 6, $|\delta_A - \delta_X|$ changes very little and $|\delta_B - \delta_C|$ even increases with temperature. Thus bilirubin and mesobilirubin are present predominantly in the fully hydrogen bonded conformations (**I**) and (**II**) (Figure 1) even at high temperatures.

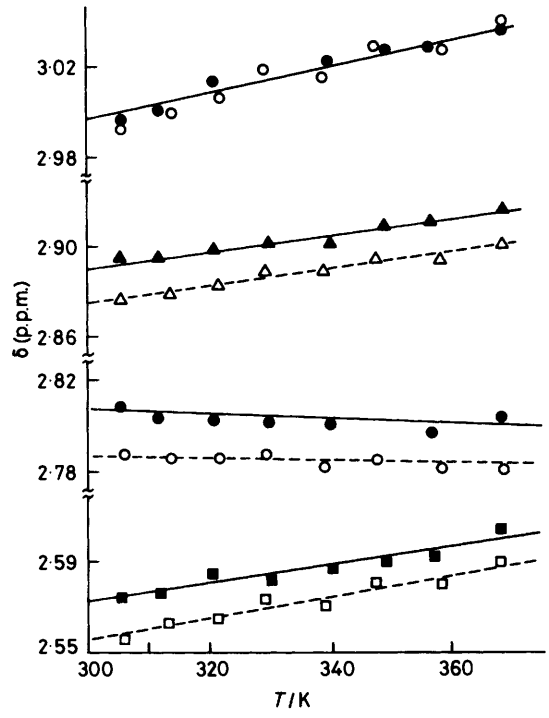

Figure 6. A plot of chemical shifts of the four propionic methylene protons of bilirubin (open symbols) and mesobilirubin (filled symbols) as a function of temperature. The differences between the two propionic acid residues are too small to be reproduced

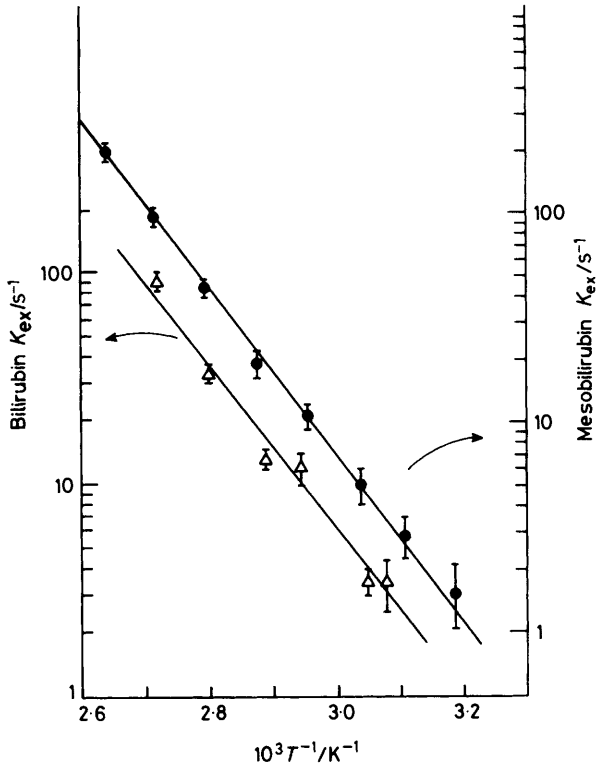

Figure 7. An Arrhenius plot of the exchange rate constants between the enantiomers of bilirubin (△) and mesobilirubin (●)

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