The Reaction of Bilirubin with Diazomethane

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Dimethoxybilirubin dimethyl ester and monomethoxybilirubin dimethyl ester were prepared by treating bilirubin with diazomethane, and the correctness of the assigned structures was proved by elemental analysis as well as by i.r. and n.m.r. spectroscopy. The phenylazo compounds derived from monomethoxybilirubin dimethyl ester were also prepared and characterized spectroscopically. Dimethoxybilirubin dimethyl ester occurs in solution as a single molecular species, unlike bilirubin dimethyl ester, which in nonpolar solvents exists as an equilibrium mixture of conformational isomers. This difference in the behaviour of the two compounds is explained by the absence of intramolecular hydrogen bonds in dimethoxybilirubin dimethyl ester, a situation that allows free rotation about the central methylene bridge, whereas in bilirubin dimethyl ester an internally hydrogen-bonded conformation can be distinguished by n.m.r. spectroscopy from a non-bonded family of rotamers. This finding is regarded as additional evidence for a newly conceived conformational structure of bilirubin and bilirubin dimethyl ester that is maximally stabilized by intramolecular hydrogen bonds. This is discussed in detail in the Appendix (Kuenzle *et al.*, 1973), which also includes a description of the molecular mechanism pertaining to the reaction of bilirubin with diazomethane.

Bilirubin reacts with diazomethane to yield, apart from bilirubin dimethyl ester, two products that have been identified by Fischer *et al.* (1941) as monomethoxybilirubin dimethyl ester and dimethoxybilirubin dimethyl ester respectively. Later, however, some doubts seem to have arisen among Fischer's pupils as to the correct assignment of structures and it was suggested that in these compounds diazomethane adds across the vinyl side chains rather than to the oxygen atoms of the outer rings (H. von Dobeneck, personal communication). It therefore seemed worthwhile to reinvestigate the products of this reaction, particularly since the n.m.r. spectrum of dimethoxybilirubin dimethyl ester may afford crucial evidence in support of a newly conceived bilirubin structure that would be maximally stabilized by intramolecular hydrogen bonds (Kuenzle *et al.*, 1973).

As an extension of previous work on the spectral properties of bilirubin derivatives (Kuenzle, 1970*a*), we now report evidence for the existence of mono-methoxy- and dimethoxy-bilirubin dimethyl ester as originally proposed by Fischer *et al.* (1941).

Experimental

Methylation products of bilirubin (*Fig.* 1)

Dimethoxybilirubin dimethyl ester (*I*) *and monomethoxybilirubin dimethyl ester* (*II*). These were prepared batchwise from 10g of commercial bilirubin

(Fluka A.-G., Buchs SG, Switzerland). All operations were carried out in the dark. Bilirubin (1 g) was dissolved by brief refluxing in chloroform (1 litre). The solution was filtered, cooled in ice, treated for 5min with approx. 0.3g of diazomethane in 30ml of diethyl ether and evaporated to dryness (evaporation was performed in a rotary evaporator at 35°C). The residue was dissolved in the minimum volume of chloroform and was chromatographed on a column (5.5cm × 60cm) of alumina (900g) (Aluminiumoxid, standardisiert, Aktivitätsstufe II-III; no. 1097; E. Merck A.-G., Darmstadt, Germany). It was observed that all batches did not possess the same activity; slightly deactivated preparations were preferred to active ones, since this speeded up chromatography and decreased oxidative degradation of the labile compounds. With chloroform as the eluent, three yellow zones appeared on the column. The first and the second fractions to emerge from the column were collected, evaporated to dryness and labelled as the crude dimethoxy- and monomethoxy-bilirubin dimethyl ester respectively.

The crude dimethoxy preparation was further purified by preparative t.l.c. on silica gel. It was streaked on to five plates (PSC-Fertigplatten, Kieselgel F_{254}; 20cm × 20cm; thickness of layer 2mm; no. 5717/0012; E. Merck A.-G.), which were developed to 5.5cm (front) with chloroform–benzene (2:1, v/v). The major yellow fraction, with R_F 0.28, was scraped from the plates, eluted with chloroform, filtered and evaporated to dryness. Crystallization
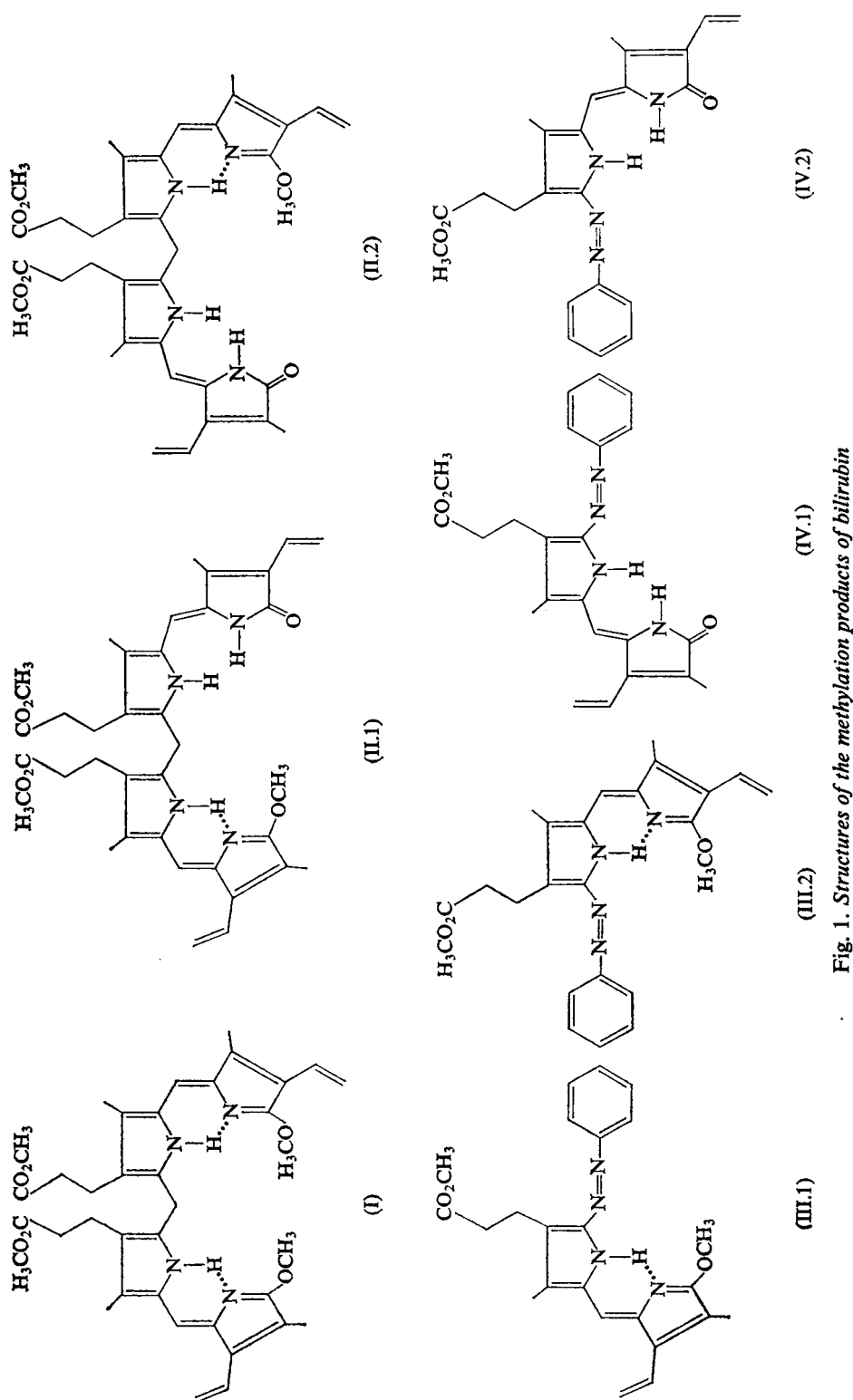
Fig. 1. *Structures of the methylation products of bilirubin*

Azo coupling of dimethoxybilirubin dimethyl ester (I) would give an azo pigment mixture consisting of the vinyl isomer (III.1) and the isovinyl isomer (III.2). However, the low yield of the starting material prevented this preparatory route. The azo pigment (III) containing both a lactim ether and an ester methoxy group was therefore prepared together with the azo pigment (IV) containing an ester function only from monomethoxybilirubin dimethyl ester (II). The latter consisted of approximately equal amounts of the isomers (II.1) and (II.2) differing in the position of the lactim methoxy substitution. On azo coupling followed by adsorption chromatography on silanized silica gel compound (II) yielded both isomers (III.1) and (III.2) as well as both isomers (IV.1) and (IV.2).

from methanol yielded 20.5 mg of *dimethoxybilirubin dimethyl ester* as shiny platelets of bright orange colour [Found: C, 69.6; H, 6.6; N, 8.8. Calc. for $C_{37}H_{44}N_4O_6$ (mol.wt. 640.79): C, 69.4; H, 6.9; N, 8.7%].

The crude monomethoxy preparation was briefly refluxed in methanol (200 ml) and then filtered. The filtrate was set aside for the preparation of the phenylazo derivatives described below. The insoluble residue was crystallized from chloroform–methanol (1:1, v/v) to give 260 mg of *monomethoxybilirubin dimethyl ester* in the form of very fine orange needles [Found: C, 69.0; H, 6.8; N, 8.8. Calc. for $C_{36}H_{42}N_4O_6$ (mol.wt. 626.77): C, 69.0; H, 6.8; N, 8.9%]. Analytical t.l.c. on silica gel (DC-Fertigplatten, Kieselgel F_{254}; 5 cm × 20 cm; thickness of layer 0.25 mm; no. 5714/0010; E. Merck A.-G.) with chloroform–acetone (20:1, v/v) as the eluent revealed that the preparation contained approximately equal amounts of the two expected isomers (II.1) and (II.2) (differing in the position of the methoxy group), but resolution on a preparative scale was not feasible.

Phenylazo derivatives of monomethoxybilirubin dimethyl ester (III, IV). The methanolic filtrate obtained after refluxing the crude preparation of monomethoxybilirubin dimethyl ester (see above) was cooled in ice to give 259 mg of coarse, olive-coloured crystals. They were dissolved in chloroform (100 ml), mixed with 2.6 ml of a freshly prepared solution of phenyldiazonium chloride (Kuenzle, 1970a) followed by 96% (v/v) ethanol (50 ml), and the coupling reaction was allowed to proceed at 20°C for 40 min. The reaction mixture was washed with three 150 ml portions of 0.2 M-acetic acid and evaporated to dryness. The residue was chromatographed on a column (4 cm × 36 cm) of silanized silica gel (250 g) (Kieselgel; particle size 0.05–0.2 mm; silanisiert für die Säulen-Chromatographie; no. 7719; E. Merck A.-G.) with carbon tetrachloride–chloroform (1:1, v/v) as the eluent. A faintly red band that moved with the solvent front and a trailing yellow zone were discarded, and the bulk of the azo pigments were then collected as two purplish-red fractions, which were clearly separated from one another. Each fraction was rechromatographed on a similar column with carbon tetrachloride–chloroform (2:1, v/v) as the eluent.

The front fraction was evaporated to dryness, and the residue was crystallized from methanol to yield 34.0 mg of fine crystals of purplish-black hue and metallic iridescence. The compound so obtained was identified as an *azo pigment containing both a lactim ether and an ester methoxy group (III)* [Found: C, 68.6; H, 6.1; N, 13.3. Calc. for $C_{24}H_{26}N_4O_3$ (mol.wt. 418.50): C, 68.9; H, 6.3; N, 13.4%].

The trailing fraction was evaporated to dryness, and the residue was dissolved in the minimum volume of redistilled xylene. The solution was filtered and evaporated to dryness at 40°C. Crystallization from methanol yielded 31.4 mg of a compound having the

same appearance as that described in the preceding paragraph. It was identified as an *azo pigment containing a single ester methoxy group (IV)*. [Found: C, 68.1; H, 6.1; N, 13.8. Calc. for $C_{23}H_{24}N_4O_3$ (mol.wt. 404.47): C, 68.3; H, 6.0; N, 13.9%].

Apparatus

N.m.r. spectra were recorded from approximately 0.05 M solutions at 100 MHz with a Varian HA-100 spectrometer. Chemical shifts (δ) are given in p.p.m. relative to tetramethylsilane = 0 (internal standard).

I.r. spectra (Nujol mulls) were recorded with a Perkin–Elmer model 21 spectrograph equipped with NaCl optics.

Optical spectra were recorded with a Beckman DB-G grating spectrometer in quartz cells of 1 cm light-path.

Results and Discussion

The analytical data presented here (see the Experimental section for elemental analysis, Fig. 2 for i.r. spectrum, and Fig. 3 and Table 1 for n.m.r. spectrum) clearly demonstrate that dimethoxybilirubin dimethyl ester has the structure (I) shown in Fig. 1, which coincides with the one originally put forward by Fischer *et al.* (1941). Two alternative structures can be excluded, although they might be conjectured to arise from the reaction of bilirubin with diazomethane: one in which the vinyl side chains of bilirubin would be transformed to cyclopropyl groups owing to addition of diazomethane (H. von Dobeneck, personal communication), and another in which substitution would occur not at the terminal oxygen atoms but at the adjacent nitrogen atom. The former structure can be ruled out by the argument that a supporting i.r. spectrum should show the presence of an amide grouping, whereas in fact it does not; further, the n.m.r. spectrum cannot be reconciled with a structure that would feature cyclopropyl in place of vinyl substituents and would allow for only two (ester) methoxy groups with four protons attached to nitrogen. On the other hand, the *N*-methyl derivative would be difficult to rule out on the strength of the available n.m.r. evidence, but, as with the structure discussed above, the lack of an i.r. absorption typical of an amide group argues strongly against this alternative. Equally strong evidence against *N*-substitution arises from steric considerations; molecular models of bilirubin show that with the introduction of an *N*-linked substituent crowding between contiguous pyrrole and pyrrolenone rings would become so severe as to force the bilirubin half-molecule from its normal planar conformation into a strained arrangement in which the pyrrolic rings

would occupy different planes. Since this would decrease the aromatic character of the molecule, such a conformation would be energetically unfavourable to the point of rendering *N*-substitution highly improbable (Kuenzle, 1973).
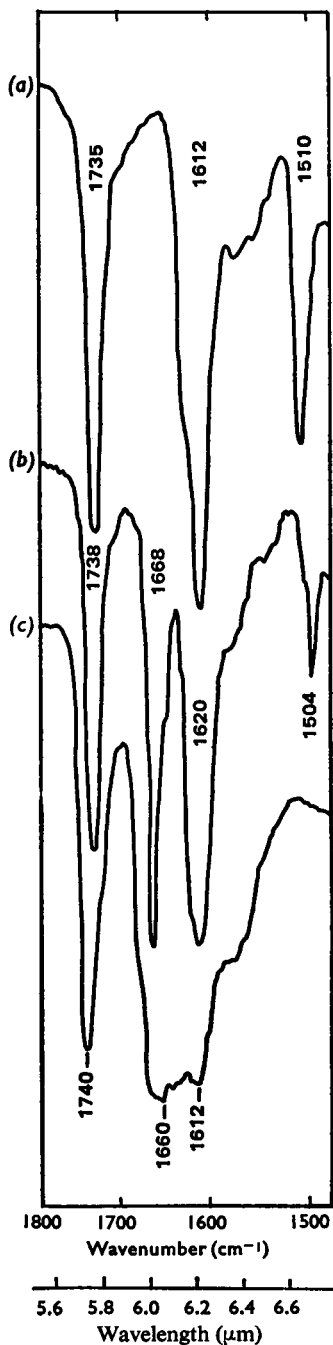
Less-direct evidence for the proposed structure of monomethoxybilirubin dimethyl ester was gained by the analyses performed, mostly because separation of the two isomers (II.1) and (II.2) was not feasible on a preparative scale. This prevented the recording of meaningful n.m.r. spectra. However, elemental analysis and i.r. spectroscopy were in agreement with the proposed structure, and the azo pigments derived from the compound were consistent with expectations. This, we consider, justifies its characterization as monomethoxybilirubin dimethyl ester, particularly when viewed in conjunction with the proof for the existence of the corresponding dimethoxy derivative.

Bilirubin dimethyl ester dissolved in non-polar solvents (chloroform and carbon tetrachloride) has been shown by n.m.r. spectroscopy to exist as two molecular species (Kuenzle, 1970*a*). In the light of a newly conceived conformational structure for bilirubin and bilirubin dimethyl ester (Kuenzle *et al.*, 1973) the two molecular forms of bilirubin dimethyl ester can now be defined as rotational isomers arising from a hindered rotation about the central methylene bridge. The factor that causes restriction of rotation is a system of intramolecular hydrogen bonds that link the ester carbonyl oxygen atoms attached to rings B and C crosswise to the amide protons of the pyrrolenone rings D and A respectively (Fig. 4). These hydrogen bonds hold the molecule in a restrained conformation sufficiently long to allow it to become registered by n.m.r. as an individual molecular species. Thus an equilibrium between hydrogen-bonded and non-bonded conformations becomes noticeable by n.m.r. spectroscopy. Since the hydrogen bonds in question involve the amide protons of the pyrrolenone rings, such a bonding cannot occur with dimethoxybilirubin dimethyl ester, which lacks the amide protons. In fact, n.m.r. spectroscopy of the latter compound dissolved in carbon tetrachloride (Fig. 3) did not give evidence of multiple conformational isomers occurring in equilibrium. On the contrary, it gave unambiguous proof for the occurrence of a single molecular species only.

Fig. 2. *I.r. absorptions due to carbonyl-stretching vibrations observed with (a) dimethoxybilirubin dimethyl ester, (b) monomethoxybilirubin dimethyl ester and (c) bilirubin dimethyl ester (Nujol mulls)*

The preparation of bilirubin dimethyl ester used has been described previously (Kuenzle, 1970*a*). Assignments are: ester vibration at 1735–1740 cm^{-1}, amide I band at 1668 and 1660 cm^{-1} respectively, and bond-stretching vibrations of the pyrrolic rings at 1612–1620 cm^{-1}. Note the absence of the amide I band in the spectrum of dimethoxybilirubin dimethyl ester.
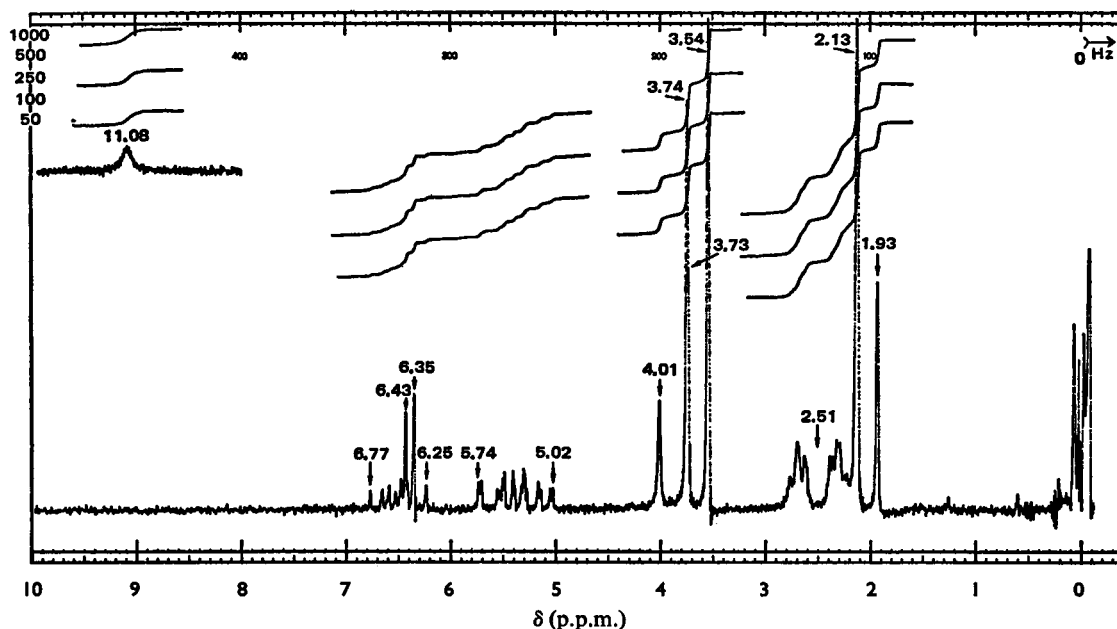
Fig. 3. *N.m.r. spectrum of dimethoxybilirubin dimethyl ester* (0.07 M *in carbon tetrachloride*)

Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). Assignments are presented in Table 1. To exclude a structure that would arise from addition of diazomethane across the vinyl side chains, thus converting the latter groups into cyclopropyl substituents, the solution was evaporated to dryness to remove tetramethylsilane and was redissolved in carbon tetrachloride containing a trace of chloroform. The spectrometer was then locked to the signal at 7.25 p.p.m. arising from the chloroform proton, and a spectrum was recorded to include the region 0–0.2 p.p.m. where signals owing to the methylene protons of cyclopropyl groups would typically be expected. However, scanning upfield to −1.4 p.p.m. did not reveal any additional resonance lines.

This we regard as an additional indication that the structures proposed for bilirubin and bilirubin dimethyl ester are essentially correct. This is the more important in this connexion since it indicates a possible molecular mechanism for the reaction of bilirubin with diazomethane and at the same time provides a rationale for the observation that dimethoxy- and monomethoxy-bilirubin dimethyl ester can only be prepared by using diazomethane when bilirubin but not bilirubin dimethyl ester is employed as the starting material. This is discussed in detail in the Appendix (Kuenzle *et al.*, 1973).

The unusually strong solvent dependency of the optical spectrum of bilirubin dimethyl ester has also been tentatively attributed to its potential to exist in multiple molecular forms (Kuenzle, 1970*a*). Although the mechanism by which a change of solvent can cause a spectral shift of up to 53 nm is not properly understood, the fact that dimethoxybilirubin dimethyl ester does not show any marked solvent dependency of its optical spectrum (Table 2) indicates that this

phenomenon might in fact be related to the conformational isomerism discussed in the preceding paragraph. Distinct solvent dependency is observed with monomethoxybilirubin dimethyl ester, although it is weak in comparison with the spectral shifts encountered with bilirubin dimethyl ester. Thus the two incompletely resolved absorption lines, occurring at approx. 420 and 450 nm respectively in either dimethyl sulphoxide or carbon tetrachloride, coalesce in chloroform to give a single broad absorption band (see Table 2); this may arise from a weak tendency to form a single intramolecular hydrogen bond of the type described above, which is insufficiently strong to resist disruption in chloroform. It therefore lacks sufficient stability to hold the molecule in a conformation suitable for initiating the hypothetical mechanism by which changes of the electronic energy levels would be induced, thus affording a possible explanation for the relatively weak solvent dependency of the optical spectrum as observed with monomethoxybilirubin dimethyl ester.

Table 1. *N.m.r. assignments of the various methylation products of bilirubin*

For the structures of the compounds listed, see Fig. 1. The numbering of carbon atoms refers to the system used previously (Kuenzle, 1970a). Chemical shifts (δ) are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). The shape and the relative area (nearest integral value) of each signal is given in parentheses. The following abbreviations are used: s, singlet; m, multiplet; A_2B_2, multiplet of A_2B_2 system. The azo pigment containing both a lactim ether and an ester methoxy group was very sparingly soluble in dimethyl sulphoxide and was even less so in deuterochloroform and carbon tetrachloride. Accordingly, the spectrum showed little detail, and some of the weaker signals were not sufficiently resolved to permit accurate analysis.

Compound ...	Dimethoxybilirubin dimethyl ester (I)	Azo pigment (III) containing both a lactim ether and an ester methoxy group	Azo pigment (IV) containing a single ester methoxy group		Assignment
Solvent ...	Carbon tetrachloride	[^{2}H$_6$]Dimethyl sulphoxide	Deuterochloroform	Carbon tetrachloride	
	Chemical shift (p.p.m.) (shape of signal, relative area)				
	1.93 (s, 3H)	2.13 (s, 4H*)	1.84 (s, 4H*)	1.75 (s, 4H*)	One of the two methyl groups on position C-2 or C-7 of the vinyl isomer†
	2.13 (s, 9H)	2.09 (s, 2H*)	2.04 (s, 8H*)	1.89 (s, 2H*)	One of the two methyl groups on position C-13 or C-17 of the isovinyl isomer†
		2.22 (s, 6H*)		2.03 (s, 6H*)	The remaining two methyl groups (one each from the vinyl and isovinyl isomer respectively†)
	2.51‡ (A_2B_2, 8H)	2.59‡ (A_2B_2, 8H)	2.85‡ (A_2B_2, 8H)	2.80‡ (A_2B_2, 8H)	Ethylene groups of propionic ester side chains on C-8 and C-12
	3.54 (s, 6H)§	3.57 (s, 12H)	3.68 (s, 6H)	3.64 (s, 6H)	Ester methoxy groups
	3.73/3.74 (two s, 6H)§		—	—	Lactim ether methoxy groups
	4.01 (s, 2H)		—	—	Methylene protons on C-10 (central methylene bridge)
	5.02–5.74 (m, 4H)	approx. 5.2–5.8 (m, ?)	5.36–6.78 (m, 8H)	5.00–6.70 (m, 8H)	Vinyl groups on C-3 and C-18
	6.23–6.77 (m, 2H)	approx. 6.2–6.8 (m, ?)			Vinyl protons on C-5 and C-15 (methene bridges a and c)
	6.35 (s, 1H)	6.13 (s, 2H)			
	6.43 (s, 1H)				
		approx. 7.1–7.8 (m, 10H)	7.00–7.56 (m, 10H)	6.70–7.50 (m, 10H)	Aromatic protons of phenylazo groups
	11.08 (s-broad, 2H)	?	10.05 (s-broad, 4H)	10.80 (s-broad, 4H)	Protons linked to nitrogen

* The integrals of all four methyl groups summed up to a total of 12 protons, which was in agreement with expectations. However, most of the individual signals exhibited integrals that could not account for multiples of three protons. This could be explained as resulting from a vinyl/isovinyl isomer ratio departing from unity. Based on the assignments of the methyl signals to the individual isomers of the azo pigment derived from bilirubin dimethyl ester as reported by Jansen & Stoll (1971) a vinyl ([IV.1]/isovinyl [IV.2]) ratio of 2:1 was calculated for the present preparation of the azo pigment that contained a single ester methoxy group. Similar asymmetric isomer distributions have been communicated by Kuenzle (1970b) and Jansen & Stoll (1971) for azo pigments derived from bile, but the present observations show that this is not a characteristic of biological material. The present spectrum of the azo pigment containing both a lactim ether and an ester methoxy group was also tentatively interpreted as reflecting a preponderance of the vinyl (III.1) over the isovinyl (III.2) isomer, although definite proof of this assignment was lacking.

† Signals of the groups cannot be specifically assigned.

‡ Centre of A_2B_2 system. The actual signals were located 0.15–0.20 p.p.m. upfield and downfield from the centre position.

§ The assignment of the singlet at 3.54 p.p.m. to the ester methoxy groups and of the two closely spaced singlets at 3.73 and 3.74 p.p.m. to the lactim ether methoxy groups was based on the reasoning that the overtly differing environments at the two ether functions would be more conducive to splitting of the methoxy signal than would the closely similar environments of the ester groupings.
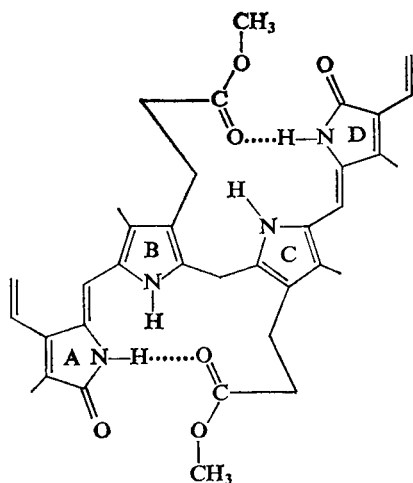
Fig. 4. *Internally hydrogen-bonded conformation of bilirubin dimethyl ester*

In non-esterified bilirubin two additional hydrogen bonds would link the carboxyl groups to the pyrrolenone oxygen atoms. Rings are labelled A–D.

A final word should be added about dimethoxy-bilirubin dimethyl ester and its optical spectrum. von Dobeneck & Brunner (1965) have classified dipyrro-methene compounds according to structure into four categories and have correlated structure with type of optical spectrum displayed. Dimethoxybilirubin dimethyl ester does not fit into this system since its structure conforms to the 'classical'-type dipyrro-methenes whereas its spectrum is reminiscent of a 'neo'-type compound (H. von Dobeneck, personal communication). This discrepancy remains unexplained at present.

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Table 2. *Optical spectra of the various methylation products of bilirubin*

For details see the Experimental section.

Compound	Solvent	$\lambda_{max.}$ (nm)	ϵ ($M^{-1} \cdot cm^{-1}$)
Dimethoxybilirubin dimethyl ester (I)	Dimethyl sulphoxide	427*	—
		456* (inflexion)	—
	Chloroform	418*	50600*
		444* (inflexion)	49200*
	Carbon tetrachloride	425*	—
		452*	—
Monomethoxybilirubin dimethyl ester (II)	Dimethyl sulphoxide	418	—
		444 (inflexion)	—
	Chloroform	430†	—
	Carbon tetrachloride	420	—
		443 (inflexion)	—
Azo pigment (III) containing both a lactim ether and an ester methoxy group	Dimethyl sulphoxide–methanolic 0.1M-acetic acid (1:9, v/v)	270	—
		278	—
		330	25100
		524‡	33800
Azo pigment (IV) containing a single ester methoxy group	Dimethyl sulphoxide–methanolic 0.1M-acetic acid (1:9, v/v)	270	12600§
		277	13400§
		327	20800§
		514	30000§

* The positions of the maxima were slightly shifted and the molar extinction coefficient was increased with respect to previous measurements performed on a less-pure preparation (Kuenzle, 1970a).

† Broad.

‡ In a previous paper (Kuenzle, 1970a) the azo pigment containing both a lactim ether and an ester methoxy group (previously labelled azo pigment MME) was described as having a flat double absorption at 506 and 520nm. This could not be substantiated in the present investigation, and apart from the observed bathochromic shift the optical spectrum did not differ appreciably from the curve obtained with the azo pigment containing a single ester methoxy group (previously labelled azo pigment ME) (see Fig. 6 in Kuenzle, 1970a). This discrepancy was attributed to impurities present in the preparation used previously.

§ Values taken from Kuenzle (1970a).

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APPENDIX

Structure and Conformation of Bilirubin

OPPOSING VIEWS THAT INVOKE TAUTOMERIC EQUILIBRIA, HYDROGEN BONDING AND A BETAINE MAY BE RECONCILED BY A SINGLE RESONANCE HYBRID

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A novel conformational structure of bilirubin is presented which obtains maximum stabilization through a system of four intramolecular hydrogen bonds. Two hydrogen bonds link oxygen and nitrogen atoms of each end ring to the contralateral carboxyl group. The proposed structure can explain a variety of uncommon features of bilirubin, and reconciles many seemingly contradictory hypotheses by accommodating them in individual structures which are mesomeric forms of one resonance hybrid. In the light of this newly conceived structure the following characteristics of bilirubin are re-evaluated: the stability of the compound, its reaction with diazomethane, the conformational behaviour of its dimethyl ester, its spectral properties, the chirality of the compound when complexed to serum albumin, and the structure of its metal chelates.

The basic structure of bilirubin was elucidated by Fischer's group (Fischer et al., 1941; Fischer & Plieninger, 1942). Nevertheless, some anomalous properties of the substance have stimulated further research. In the first instance, Fischer's original proposal that bilirubin exists as the bis-lactim had to be modified on the strength of evidence gained from other 'α-hydroxypyrroles' (Plieninger & Decker, 1956; Plieninger et al., 1962) and from spectra of bilirubin and its derivatives (Gray et al., 1961; von Dobeneck & Brunner, 1965; Kuenzle, 1970a). Thus today it is common usage to represent bilirubin with the two outer rings in the lactam configuration. An exception to this is a recent claim for the prevalence of the bis-lactim form (Nichol & Morell, 1969), but this inference was based on misinterpreting the n.m.r. spectrum of bilirubin (Kuenzle, 1970a). However, present-day spectroscopy cannot exclude the occurrence of very small quantities of a bis-lactim tautomer at equilibrium. This has been invoked in explanation of mono- and di-methoxy derivatives forming

when bilirubin is esterified with diazomethane (Kuenzle, 1970a).

Prompted by the experience that bilirubin is much less prone to oxidation than both its anion and its naturally occurring acyl glycosides, Fog and co-workers (Fog & Bugge-Asperheim, 1964; Fog & Jellum, 1963) have suggested that this may be due to the stabilizing effect of intramolecular hydrogen bonds existing between the carboxylic acid groups and the pyrrole nitrogen atoms of the inner rings. Other types of hydrogen bonds have been implicated by Brodersen et al. (1967), Nichol & Morell (1969), Kuenzle (1970a) and Hutchinson et al. (1971). To support the various hypotheses, the unusually low i.r. frequencies associated with the carboxyl (1680cm⁻¹) and amide (1645cm⁻¹) functions have been invoked (Fog & Jellum, 1963; Brodersen et al., 1967; Nichol & Morell, 1969; Hutchinson et al., 1971). However, neither of the proposed structures has proved completely convincing, either because in devising the structure steric requirements have been