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Bilirubin Conjugates of Human Bile

NUCLEAR-MAGNETIC-RESONANCE, INFRARED AND OPTICAL SPECTRA OF MODEL COMPOUNDS

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N.m.r., i.r. and optical spectra of model compounds were recorded. These were to help in elucidating the structures of the phenylazo derivatives of bilirubin conjugates isolated from human bile. Model compounds included commercial and human bile bilirubin, mesobilirubin, bilirubin dimethyl ester, dimethoxybilirubin dimethyl ester and the corresponding phenylazo derivatives. The phenylazo derivative of vinylneoxanthobilirubinic acid was also investigated. All compounds were of the type IXα, and no other isomer could be detected with the spectroscopic methods employed. The compounds crystallize as the lactams, except for dimethoxybilirubin dimethyl ester and its phenylazo derivative, which are held in the lactim ether configuration. With all other compounds no tautomeric forms other than the lactams could be detected, although small proportions of bilirubin must exist as the lactim. Bilirubin does not form a betaine, a structure that has been proposed by von Dobeneck & Brunner (1965) to explain the bathochromic shift of its optical spectrum as compared with the expected position of the absorption maximum at 420nm. However, this shift to 453nm can be explained on the basis of internal hydrogen bonds occurring between the carboxylic protons and the pyrrole rings of bilirubin, as proposed by Fog & Jellum (1963), and new evidence for such a bonding has been accumulated. The bilirubin sulphate described by Watson (1958), which is formed by treatment of bilirubin with concentrated sulphuric acid and acetic anhydride, was also investigated. The main product of this reaction was isolated as its phenylazo derivative, and was shown to be 3,18-di(ethylidene sulphate)-2,7,13,17-tetramethylbiladiene-*ac*-8,12-dipropionic acid. The reaction leading to this compound is an addition of sulphuric acid to the vinyl side chains of bilirubin according to Markownikoff's rule.

The isolation of various phenylazo derivatives of bilirubin from human hepatic bile was reported in the preceding paper (Kuenzle, 1970*a*). Structure elucidation was attempted, and in the case of the major compounds partial structures were obtained (Kuenzle, 1970*b*). Spectra of model compounds, i.e. bilirubin and some of its derivatives, greatly facilitated the structural analysis of the isolated pigments. N.m.r., i.r. and optical spectra of the model compounds used for that purpose are presented in this paper. N.m.r. spectra of the following bilirubinoid compounds have been published previously: bilirubin (Nichol & Morell, 1969), bilirubin dimethyl ester (Nichol & Morell, 1969), biliverdin dimethyl ester (Nomura, Gaudemer, Barbier & Tsuchiya, 1966; Cole, Chapman & Siegelman, 1968) and mesobiliverdin dimethyl ester (Cole *et al.* 1968).

It has been known since the work of Fischer and co-workers (Fischer, Plieninger & Weissbarth, 1941; Fischer & Plieninger, 1942) that bilirubin from human and animal sources is of the type IXα (for a definition of the 52 conceivable isomers of bilirubin, see Fischer & Orth, 1937). Investigations as to the possible occurrence of isomers other than IXα have led to the conclusion that dog bile and commercial bilirubin contain such an isomer in very small proportions together with bilirubin IXα (Petryka, 1966). The results of the present investigation are not affected by the occurrence of this unidentified isomer, since the reported amounts are too small for detection with the spectroscopic methods employed.

Bilirubin and its derivatives, when treated with diazonium salts, yield purplish-red azo pigments.

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The reaction is only observed with bile pigments that possess a central methylene bridge. Consequently biliverdin does not undergo this reaction. On the other hand, the reaction is also observed with neo- and isoneo-xanthobilirubinic acid, as well as with vinyl- and isovinyl-neoxanthobilirubinic acid (Fischer *et al.* 1941; Fischer & Plieninger, 1942). These substrates are oxodipyrrylmethenes with an unsubstituted α-position on one of the pyrrole rings. The reaction of bilirubinoid compounds with diazonium salts has been investigated by Fischer's group (Fischer & Haberland, 1935; Fischer *et al.* 1941) and by Overbeek, Vink & Deenstra (1955). The reaction proceeds in two steps, during which one molecule of bilirubin is transformed into two molecules of azo pigment. The first step consists of a random attack of the diazonium ion on either one of the α-positions adjacent to the central methylene group of bilirubin. This results in the splitting of the molecule on the corresponding side of the central methylene group with the consequent release of two unequal fragments. One of the fragments is an oxodipyrrylmethene azo pigment, whereas the other is believed to be an oxodipyrrylmethene carbinol. In the second step of the reaction the oxodipyrrylmethene carbinol is attacked by another diazonium ion, being thus transformed into a second molecule of oxodipyrrylmethene azo pigment; the carbinol group is lost in this process. In the special case of bilirubin IXα azo coupling of this unsymmetrical compound yields two isomeric azo pigments. As a result of the random attack of the diazonium ion on either side of the central methylene group equimolar proportions of the two isomeric azo pigments are found at any time in the reaction mixture. Thus coupling of bilirubin IXα with phenyldiazonium chloride yields an equimolar mixture of the phenylazo derivatives of vinyl- and isovinyl-neoxanthobilirubinic acid (Fischer *et al.* 1941).

In this paper the following designations have been used for the azo pigments: azo pigment A_1, mixture of the isomeric phenylazo derivatives of bilirubin IXα; azo pigment $A_{1.1}$, phenylazo derivative of vinylneoxanthobilirubinic acid; azo pigment $A_{1.2}$, phenylazo derivative of isovinylneoxanthobilirubinic acid; azo pigment MESO, mixture of the isomeric phenylazo derivatives of mesobilirubin IXα; azo pigment ME, mixture of the isomeric phenylazo derivatives of bilirubin IXα dimethyl ester; azo pigment MME, mixture of the isomeric phenylazo derivatives of dimethoxybilirubin IXα dimethyl ester.

EXPERIMENTAL

N.m.r. spectra of the model compounds (0.05M solutions) were recorded at 100MHz with a Varian HR-100 spectrometer. Solvents were [$^2H_6$]dimethyl sulphoxide, [^2H]chloroform and carbon tetrachloride. Chemical shifts are given in p.p.m. (±0.03) relative to tetramethylsilane = 0 (internal standard). Coupling constants, $|J|$, are given in Hz.

I.r. spectra (Nujol mulls) were recorded with a Perkin–Elmer model 21 spectrograph equipped with NaCl optics. Near-i.r. spectra were recorded in dry carbon tetrachloride (0.2mM solutions) in quartz cells of 10cm light-path with the use of a Beckman DK2 spectrophotometer.

Qualitative and quantitative optical spectra were recorded in quartz cells of 1cm light-path with the use of a Beckman DK2 and a Unicam SP.500 spectrophotometer respectively. Phenylazo derivatives were measured in dimethyl sulphoxide–methanolic 0.1M-acetic acid (1:9, v/v), and various solvents were used with the other compounds.

Model compounds were prepared from commercial bilirubin or from derivatives thereof, except in one instance, where vinylneoxanthobilirubinic acid methyl ester was used as the starting material. Commercial bilirubin was purchased either from Pfanstiehl Laboratories, Waukegan, Ill., U.S.A., or from Fluka A.G., Buchs SG, Switzerland, and was purified before use. Vinylneoxanthobilirubinic acid methyl ester was a gift from Professor H. Plieninger, Heidelberg, Germany. For the structures of the compounds prepared see Fig. 1.

Preparation of model compounds

Bilirubin (I). Commercial bilirubin was purified by passage through anhydrous Na_2SO_4, followed by crystallization from chloroform (Fog, 1964) [Found: C, 67.5; H, 6.3; N, 9.4. Calc. for $C_{33}H_{36}N_4O_6$ (mol.wt. 584.65): C, 67.8; H, 6.2; N, 9.6%].

Bilirubin from human hepatic bile was obtained by the method of Clarke (1965), but crystallization from pyridine was not effected. Instead, the crude product was purified by the method of Fog (1964) [Found: C, 66.1; H, 6.3; N, 9.4. Calc. for $C_{33}H_{36}N_4O_6$ (mol.wt. 584.65): C, 67.8; H, 6.2; N, 9.6%].

Mesobilirubin (II). Mesobilirubin was prepared from bilirubin (I) by hydrogenation essentially as described by Fischer *et al.* (1941). A 10% Pd–$BaSO_4$ catalyst was used. The reaction was stopped when uptake of hydrogen had ceased to occur. The crude product obtained on evaporation of the solvent (throughout this work evaporation of solvents was performed in a rotary evaporator at 35°C unless otherwise stated) was dissolved in chloroform and washed twice with water. The organic layer was evaporated to dryness. The residue was again dissolved in chloroform, and was purified by passage through anhydrous Na_2SO_4 with chloroform as eluent (Fog, 1964). Crystallization was effected from chloroform–methanol (1:1, v/v) [Found: C, 67.1; H, 7.0; N, 9.4. Calc. for $C_{33}H_{40}N_4O_6$ (mol.wt. 588.68): C, 67.3; H, 6.8; N, 9.5%].

Bilirubin dimethyl ester (III) and dimethoxybilirubin dimethyl ester (IV). Bilirubin (I) dissolved in chloroform was treated with diazomethane and the mixture of products was chromatographed on alumina (Fischer *et al.* 1941). The three fractions, consisting of dimethoxybilirubin dimethyl ester, monomethoxybilirubin dimethyl ester [the two isomeric monomethoxybilirubin dimethyl esters emerge from the column in a single fraction; they

can be separated by subsequent t.l.c. on glass plates (20cm × 20cm) each coated with 7g of silica gel G ('nach Stahl'; E. Merck A. G., Darmstadt, Germany) suspended in 15ml of 0.3M-sodium acetate; chloroform–ethyl acetate (5:3, v/v) is used as the eluent] and bilirubin dimethyl ester respectively, were collected in that order and evaporated to dryness. Bilirubin dimethyl ester (III) was crystallized twice from propan-2-ol [Found: C, 68.4; H, 6.7; N, 9.2. Calc. for $C_{35}H_{40}N_4O_6$ (mol.wt. 612.73): C, 68.6; H, 6.6; N, 9.1%].

Dimethoxybilirubin dimethyl ester (IV) was crystallized from methanol. No elemental analysis was performed because sufficient amounts of this substance were not obtained.

Deuterated bilirubin dimethyl ester. A 15mg portion of bilirubin dimethyl ester (III) was dissolved in 2ml of dry chloroform. The solution was vigorously shaken with 3.3ml of deuterium oxide for 15min in the dark. The aqueous layer was drawn off, and the deuterium exchange was repeated twice with fresh deuterium oxide. The final chloroform layer was evaporated to dryness with a stream of dry N_2 at 60°C, and the residue of deuterated bilirubin dimethyl ester was dried for 30h over P_2O_5 at 0.001mmHg at 20°C. Deuterium back-exchange was performed as described above, but water was substituted for deuterium oxide.

Azo pigment A_1 (V; azo pigment A_1 consists of equimolar proportions of the two isomers V.1 and V.2). A chilled solution of phenyldiazonium chloride was freshly prepared from 0.4g of $NaNO_2$ in 5.0ml of water, and 0.5ml of aniline (freshly distilled) in 9.0ml of 1.5M-HCl. A 2ml portion of this reagent followed by 150ml of 96% (v/v) ethanol was added to a solution of 200mg of bilirubin (I) in 300ml of chloroform. The reaction mixture was left to stand for 30min at 20°C in the dark. The purple solution was washed with three 150ml portions of 0.2M-acetic acid and evaporated to dryness. The residue was further dried in a high vacuum. It was then dissolved in the minimum volume of chloroform and passed through a column (3.1cm × 32cm) of anhydrous Na_2SO_4 with chloroform as eluent. A dark-red band moved with the solvent front. It was collected and evaporated to dryness. The residue was dissolved in the minimum volume of methanol, and was filtered to remove some unchanged bilirubin. The filtrate was evaporated to dryness, and the methanol extraction of the residue was repeated twice. The final residue was dissolved in the minimum volume of chloroform and was again passed through anhydrous Na_2SO_4. The pigmented fraction was collected and evaporated to dryness. The residue was crystallized from methanol–water (3:1, v/v) to yield 70mg of azo pigment A_1 [Found: C, 68.0; H, 6.0; N, 14.5. Calc. for $C_{22}H_{22}N_4O_3$ (mol.wt. 390.43): C, 67.7; H, 5.7; N, 14.4%].

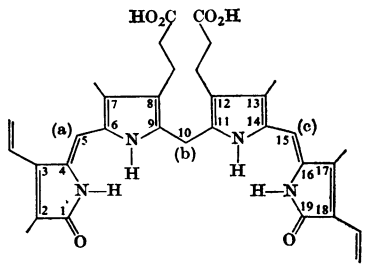
Azo pigment $A_{1.1}$ (V.1). Azo pigment $A_{1.1}$ was prepared from vinylneoxanthobilirubinic acid methyl ester (Fischer & Plieninger, 1942). A 30mg portion of the ester was dissolved in 50ml of methanol. Then 50ml of 0.2M-KOH was added, and saponification was effected by refluxing for 1.5h. The reaction mixture was cooled in ice and acidified with 2M-HCl. The yellow pigment was exhaustively extracted with chloroform. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in 40ml of chloroform. Azo coupling was performed and the reaction mixture was treated essentially as described for azo pigment A_1; the extraction with methanol and the second passage through anhydrous Na_2SO_4 were not performed. Crystallization from methanol yielded 28mg of azo pigment $A_{1.1}$ [Found: C, 68.3; H, 6.3; N, 13.3. Calc. for $C_{22}H_{22}N_4O_3$ (mol.wt. 390.43): C, 67.7; H, 5.7; N, 14.4%].

Azo pigment MESO (VI). Mesobilirubin (II) (245mg) in chloroform (500ml) was coupled with phenyldiazonium chloride, and was treated essentially as described for azo pigment A_1, but the second passage through anhydrous Na_2SO_4 was omitted. Instead, the final methanol extract was evaporated to dryness, and the residue was crystallized from methanol–water (3:1, v/v). The crystals were collected and dried. They were then dissolved in the minimum volume of methanol, and the solution was filtered to remove some residual mesobilirubin. The filtrate was evaporated to dryness and the methanol extraction was repeated. Recrystallization from methanol–water (3:1, v/v) yielded 95mg of azo pigment MESO [Found: C, 67.2; H, 6.2; N, 14.1. Calc. for $C_{22}H_{24}N_4O_3$ (mol.wt. 392.44): C, 67.3; H, 6.2; N, 14.3%].

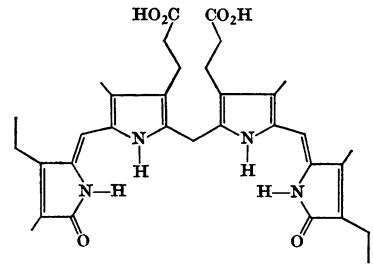
Azo pigment ME (VII). Bilirubin dimethyl ester (III) (500mg) in chloroform (300ml) was coupled with phenyldiazonium chloride essentially as described for azo pigment A_1. The reaction mixture was washed with three 150ml portions of 0.2M-acetic acid, and the organic layer was evaporated to dryness. The residue was dissolved in 30ml of carbon tetrachloride, and 30ml of hexane was added to bring about partial precipitation of a brown impurity. The precipitate was removed by filtration and the filtrate was passed through a column (4.1cm × 48cm) of anhydrous Na_2SO_4 with carbon tetrachloride–hexane (1:1, v/v) as eluent. The red front band was poorly separated from a trailing fraction of yellow impurities. The former was collected and evaporated to dryness. The residue was again passed through a column (6.3cm × 48cm) of anhydrous Na_2SO_4, and the azo pigment fraction was collected and evaporated to dryness. The residue was crystallized from methanol. Recrystallization from carbon tetrachloride–hexane yielded a mixture of yellow and purplish-black crystals. These were removed by filtration and discarded. The filtrate was evaporated to dryness and the residue was dissolved in the minimum volume of xylene. The solution was filtered and evaporated to dryness at 40°C. Crystallization from methanol yielded 48mg of azo pigment ME [Found: C, 68.0; H, 6.2; N, 13.6. Calc. for $C_{23}H_{24}N_4O_3$ (mol.wt. 404.47): C, 68.3; H, 6.0; N, 13.9%].

Azo pigment MME (VIII). Dimethoxybilirubin dimethyl ester (IV) (0.2mg) in chloroform (20ml) was coupled with phenyldiazonium chloride. The reaction mixture was washed three times with 0.2M-acetic acid and the organic layer was evaporated to dryness. The residue was dissolved in 1ml of carbon tetrachloride, and 1.5ml of hexane was added. The solution was passed through a column (1.8cm × 15cm) of anhydrous Na_2SO_4 with carbon tetrachloride–hexane (2:3, v/v) as eluent. The azo pigment fraction was collected and evaporated to dryness. The residue of azo pigment MME was not purified further, and elemental analysis was not performed.

Azo pigment S_5 (IX). Azo pigment S_5 was prepared from bilirubin (I) essentially by a modification (Gregory & Watson, 1962) of the original method of Watson (1958).



(I)



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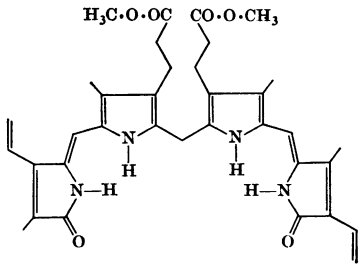

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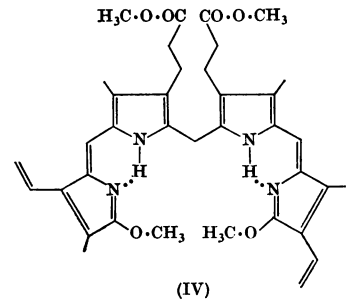

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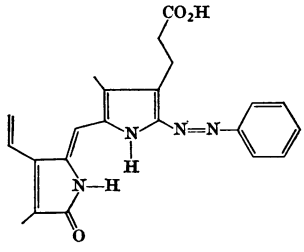
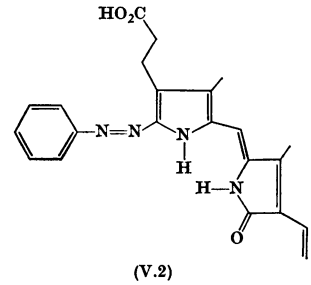


(V.1)



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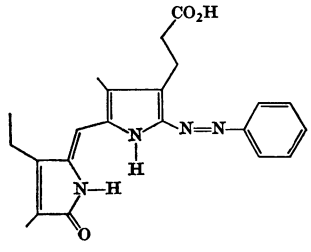

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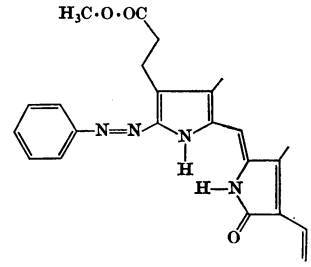

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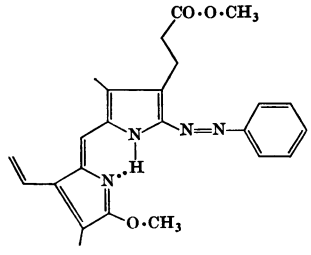

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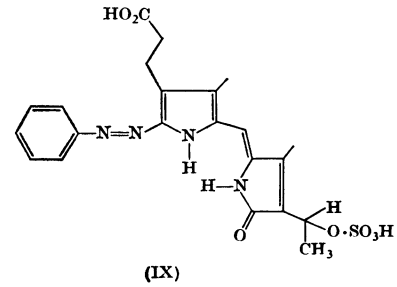


(IX)

Fig. 1.

To a solution of 4g of bilirubin in 7.6 litres of chloroform were added with shaking 3 litres of acetic anhydride–conc. H_2SO_4 (160:1, v/v). After 1min 1kg of crushed ice was added, and the mixture was shaken to extract the polar pigments into the aqueous layer. To the organic layer were added 2 litres of the above acetic anhydride–H_2SO_4 reagent followed by 1kg of ice, and the extraction was repeated. The combined aqueous extracts were adsorbed on a column (12cm × 15cm) prepared from 2kg of alumina (neutral, Brockmann grade II–III). Elution with 0.1M-NaOH resulted in the separation of three pigmented fractions, which appeared from top to bottom as one red, one brownish-yellow and one green band. Elution was discontinued when the separation seemed almost complete. The upper two fractions were scraped from the column. They were eluted with 8.7 litres of 0.1M-NaOH and filtered by suction. The filtrate was acidified to pH5 with acetic acid. Then 40ml of butan-1-ol was added followed by 15ml of phenyldiazonium chloride solution (see preparation of azo pigment A_1). After 30min the reaction mixture was exhaustively extracted with butan-1-ol, and the combined extracts were washed three times with a total of 10 litres of 0.2M-acetic acid. The butanol layer was evaporated to dryness in a rotary evaporator at 25°C by azeotropic distillation with water, to yield 180mg of a crude azo pigment mixture. The latter was subjected to reverse-phase partition chromatography on silicone-treated Celite with a solvent system prepared from butan-1-ol and 5mM-sodium–potassium phosphate buffer, pH6.0 (Kuenzle, 1970*a*). A column (6.3cm × 22cm) was prepared from 250g of silicone-treated Celite as described in the preceding paper (Kuenzle, 1970*a*). The crude azo pigment mixture was dissolved by shaking with twelve 10ml portions of the aqueous layer (mobile phase) of the above solvent system. The combined solutions were filtered and applied to the column. On elution with mobile phase a multitude of differently coloured fractions appeared. Five of these had the purplish-red colour typical of the phenylazo derivatives of bilirubin compounds. They were numbered from top to bottom and were designated as azo pigment S_1 to azo pigment S_5. The last was by far the most intense and was estimated to account for approx. 95% of the total azo pigments present. The eluate of azo pigment S_5 was collected and exhaustively extracted with butan-1-ol. The combined extracts were washed with three 400ml portions of 0.2M-acetic acid, and the washings were back-extracted with two 50ml portions of butan-1-ol. The combined butanol extracts were evaporated to dryness as described above to yield 75mg of crude azo pigment S_5. The latter was purified by reverse-phase partition chromatography on a column (4.4cm × 38cm) of silicone-treated Celite (200g) with the use of a solvent system prepared from octan-1-ol–di-isopropyl ether–ethyl acetate–methanol–0.2M-acetic acid (1:2:2:3:4, by vol.) (Kuenzle, 1970*a*). A single azo pigment band moved through the column. It was collected and concentrated in a rotary evaporator at 25°C. The resulting octanol concentrate was evaporated to dryness at 0.001mmHg at 35°C. The residue was dissolved in a mixture of 200ml of butan-1-ol and 250ml of 0.2M-acetic acid and exhaustively extracted with butan-1-ol. The combined extracts were evaporated to dryness by azeotropic distillation with water. The residue was dissolved in 30ml of methanol and filtered. To the filtrate was added slowly with shaking 150ml of ethyl acetate followed by 150ml of hexane, and the solution was slowly concentrated in a rotary evaporator at 20°C to precipitate the pigment partially. To the suspension was added dropwise with shaking 6vol. of dry ether. The precipitate was collected by filtration and washed with dry ether. The purplish-black powder was found to be highly hygroscopic, and was dried for 24h at 0.001mmHg at 20°C over P_2O_5. The yield was 47mg of azo pigment S_5.

Fig. 1. Structures of the model compounds investigated. All model compounds were of the type IXα. Structures of the following compounds are shown: bilirubin (I), mesobilirubin (II), bilirubin dimethyl ester (III), dimethoxybilirubin dimethyl ester (IV), azo pigment A_1 (V) consisting of equimolar proportions of azo pigment $A_{1.1}$ (V.1) and azo pigment $A_{1.2}$ (V.2), azo pigment MESO (VI), azo pigment ME (VII), azo pigment MME (VIII) and azo pigment S_5 (IX). Both isomers (V.1 and V.2) obtained on azo coupling of bilirubin IXα are shown. Similarly the preparation of compounds (VI) to (IX) from type IXα bilirubinoids yields both isomers. However, only one isomer is depicted in each case. The configuration at the methene bridges (*a*) and (*c*) as shown for all compounds, is the same as in protoporphyrin, from which bilirubin is derived biosynthetically. The nomenclature and the numbering of atoms follows the system suggested by Dolphin *et al.* (1966). In this system bilirubin is described as 2,7,13,17-tetramethyl-3,18-divinylbiladiene-*ac*-8,12-dipropionic acid.

RESULTS AND DISCUSSION

Spectra of model compounds of known structures. All model compounds investigated, except for dimethoxybilirubin dimethyl ester and its phenylazo derivative, crystallize as the lactams, in agreement with the structures presented in Fig. 1. This is evident from their i.r. spectra, which show amide carbonyl absorptions in the range 1640–1655cm^{-1}. Details of the i.r. spectrum of bilirubin are not presented in this paper since they have already been described by many investigators (Henry, Jacobs & Chiamori, 1960; Fog & Jellum, 1963; Newbold & LeBlanc, 1964; Suzuki & Toyoda, 1967; Nichol & Morell, 1969). The i.r. spectrum of mesobilirubin has also been published (Fog & Jellum, 1963); and the i.r. spectra of bilirubin dimethyl ester and of mesobilirubin dimethyl ester (Fog & Jellum, 1963) are virtually identical. Dimethoxybilirubin dimethyl ester shows a weak N–H absorption at 3245cm^{-1} and a sharp ester carbonyl band at 1735cm^{-1}. No absorption is found in the region of 1650cm^{-1} since the amide carbonyl group is missing in this compound. Otherwise the i.r. spectra of dimethoxybilirubin dimethyl ester and of bilirubin dimethyl ester are virtually identical. The i.r. spectrum of azo pigment A_1 (Fig. 2) and of azo pigment $A_{1.1}$ are identical, and

are almost indistinguishable from the spectrum of azo pigment MESO. The i.r. spectrum of azo pigment ME shows an ester carbonyl band at 1735 cm^{-1} and a shift of the amide carbonyl absorption to 1692 cm^{-1}, but otherwise it is very similar to the spectrum of azo pigment A_1.

N.m.r. spectra were recorded in various solvents. Those recorded from [$^2H_6$]dimethyl sulphoxide solutions of bilirubin, mesobilirubin, bilirubin dimethyl ester, azo pigment A_1 and azo pigment $A_{1.1}$ (Figs. 3*a*–3*d*) are discussed first. Intercomparison of the five spectra, and deuterium exchange experiments (footnote ** in Table 1), greatly helped in the assignment of signals as given in Table 1. A spectrum of bilirubin dimethyl ester in [^2H]chloroform (inset of Fig. 3*c*) confirmed the presence of an A_2B_2 multiplet, which was buried beneath other signals when the spectrum was

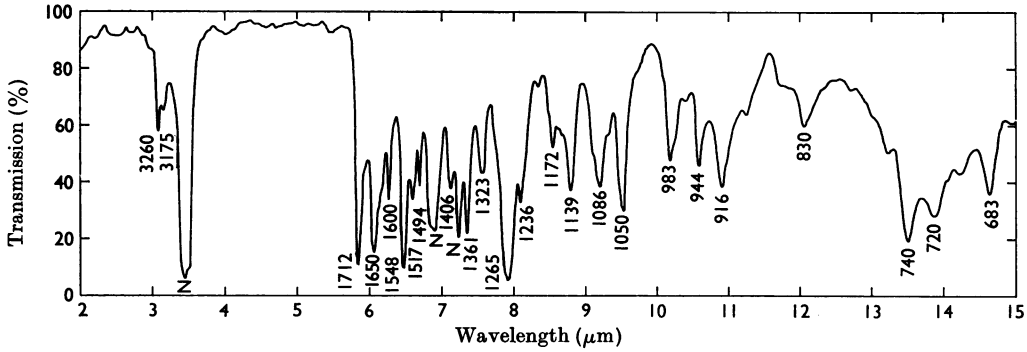
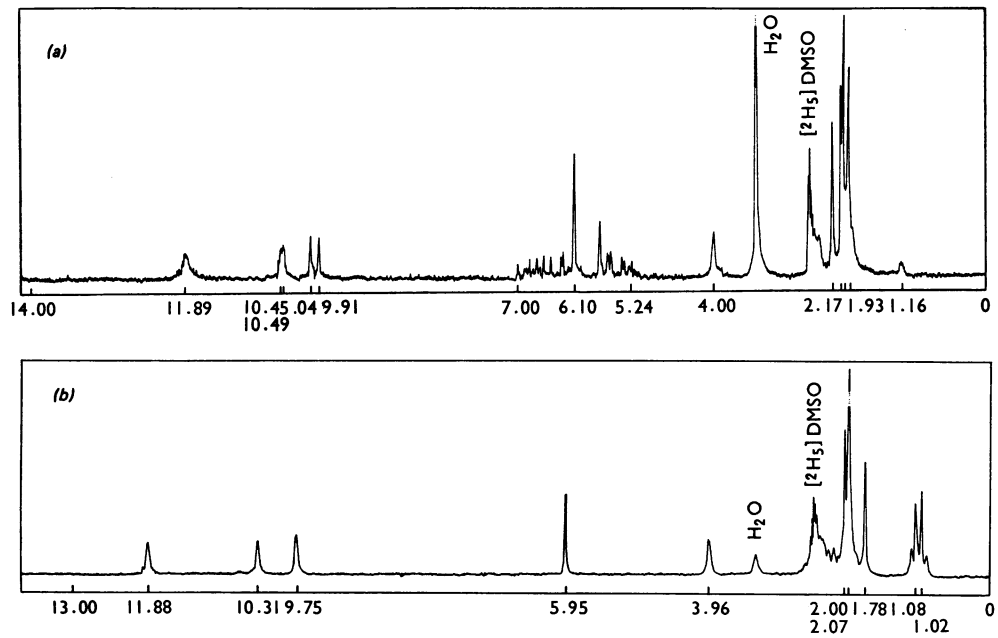


Fig. 2. I.r. spectrum of azo pigment A_1 (Nujol mull). Positions of bands are given in cm^{-1}. Nujol absorptions are marked with N. Assignments are: N–H stretching frequencies of pyrrole and α-pyrrolone rings at 3260 and 3175 cm^{-1}; the CO·O–H stretching mode is probably buried beneath the N–H and Nujol absorptions; carbonyl stretching frequency of carboxyl group at 1712 cm^{-1}; carbonyl stretching frequency of α-pyrrolone (amide I band) at 1650 cm^{-1}. The i.r. spectra of azo pigment A_1 and azo pigment $A_{1.1}$ are identical.



Figs. 3*a* and 3*b*.

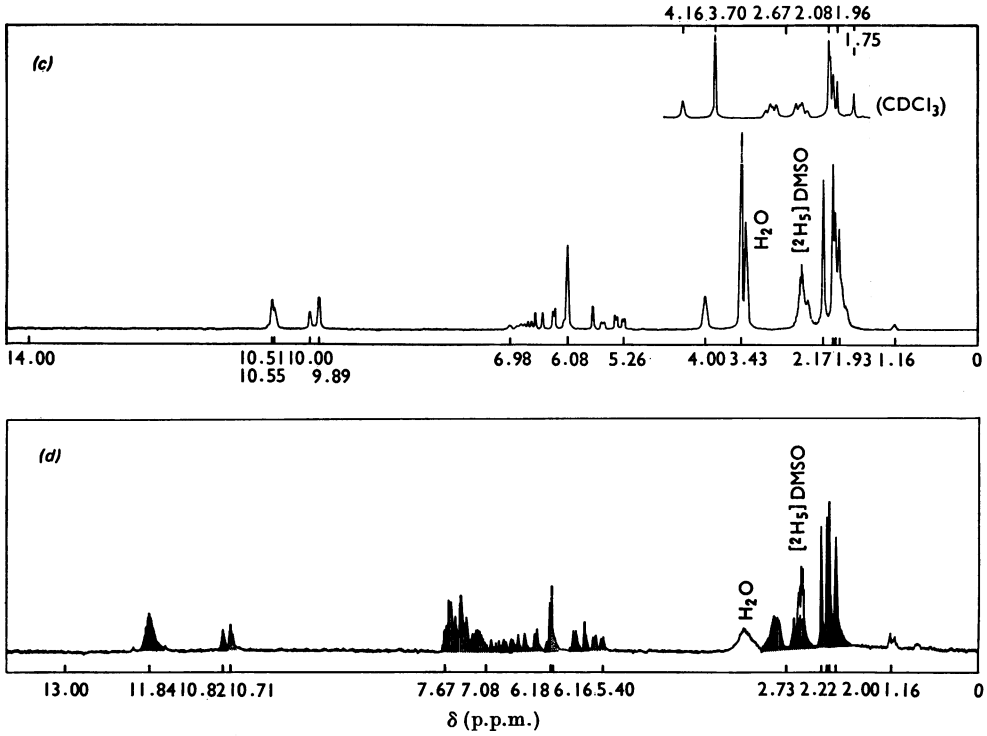


Fig. 3. N.m.r. spectra of bilirubin (*a*), mesobilirubin (*b*), bilirubin dimethyl ester (*c*) and azo pigment A_1 and azo pigment $A_{1.1}$ (*d*) (0.05M in [$^2H_6$]dimethyl sulphoxide). Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). For the structures of the compounds see Fig. 1. Assignments are given in Table 1. In (*a*), (*c*) and (*d*) the small peak at 1.16 p.p.m. is believed to be due to an impurity. The inset in (*c*) shows part of the spectrum of bilirubin dimethyl ester in [^{2}H]chloroform. It is presented here to demonstrate the multiplet of the A_2B_2 system (centred at 2.67 p.p.m.) assigned to the ethylene groups of both propionic ester side chains. Further details of this spectrum are presented in Table 2 and are discussed in the text. The five methyl singlets at 1.75, 1.96, 2.03, 2.06 and 2.08 p.p.m. should be noted. In (*d*) the spectrum of azo pigment A_1 is presented. This compound consists of equimolar proportions of azo pigment $A_{1.1}$ and azo pigment $A_{1.2}$. Therefore the spectrum of azo pigment $A_{1.1}$ (black areas) is shown as part of the spectrum of azo pigment A_1. The spectrum of azo pigment $A_{1.2}$ (stippled areas) was not recorded but could be deduced from the spectra of azo pigment A_1 and azo pigment $A_{1.1}$. Signals pertaining to both azo pigment $A_{1.1}$ and azo pigment $A_{1.2}$ are shown as striped areas. Signals of the solvents water (H_2O) and [$^2H_5$]dimethyl sulphoxide ([$^2H_5$]DMSO) are shown in white.

recorded in [$^2H_6$]dimethyl sulphoxide. Conclusive interpretation of the spectrum of azo pigment A_1 also required the recording of an additional spectrum, which confirmed the assignment of signals pertaining to azo pigment $A_{1.1}$ and azo pigment $A_{1.2}$ respectively (footnote ¶ in Table 1). The present assignments are supported further by their agreement with those reported for biliverdin dimethyl ester (Nomura *et al.* 1966; Cole *et al.* 1968) and mesobiliverdin dimethyl ester (Cole *et al.* 1968). On the other hand, Nichol & Morell (1969) give a different interpretation of the n.m.r. spectrum of bilirubin. This discrepancy is discussed below.

N.m.r. spectra were recorded from commercial bilirubin and from bilirubin obtained from human hepatic bile. Spectra of both samples were found to be identical. Neither one gave evidence of the existence of an isomer other than IXα. Such an isomer has been reported to occur in dog bile and in commercial bilirubin (Petryka, 1966), although in such small proportions with bilirubin IXα that it would not be detected by n.m.r. spectroscopy. The occurrence of an isomer other than IXα has been proposed on the basis of oxidative degradation studies. On oxidation of bilirubin with alkaline potassium permanganate, small amounts of 2,3,5-tricarboxy-4-methylpyrrole have been obtained (Petryka, 1966). This compound would be expected to arise from

Table 1. *N.m.r. assignments of bilirubin, mesobilirubin, bilirubin dimethyl ester, azo pigment A_1 and azo pigment $A_{1.1}$ (0.05M in [$^2H_6$]dimethyl sulphoxide)*

For the structures of the compounds listed see Fig. 1. The numbering of carbon atoms refers to the system used for bilirubin even when phenylazo derivatives are described. Chemical shifts are given in p.p.m. (±0.03) relative to tetramethylsilane = 0 (internal standard). The shape and the relative area (nearest integral value) of each signal are given in parentheses. The following abbreviations are used: s, singlet; t, triplet; m, multiplet; A_2B_2, multiplet of A_2B_2 system.

Compound ...	Chemical shift (p.p.m.) (shape of signal, relative area)					
	Bilirubin (I)	Mesobilirubin (II)	Bilirubin dimethyl ester (III)	Azo pigment A_1 (V)	Azo pigment $A_{1.1}$ (V.1)	Assignment
	–	1.02 (t*, 3H) 1.08 (t*, 3H)	—	—	—	Methyl groups of ethyl side chains on C-3 and C-18†
	–	~2.00–2.60‡	—	—	—	Methylene groups of ethyl side chains on C-3 and C-18†
	1.93 (s, 3H)	1.78 (s, 3H)	1.93 (s, 3H)	2.00 (s, 3H)	2.00 (s, 3H)	Methyl groups on C-2 and C-7†
	2.00 (s, 3H)	2.00 (s, 6H)	2.00 (s, 3H)	2.10 (s, 3H)	2.10 (s, 3H)	
	2.03 (s, 3H)		2.03 (s, 3H)	2.13 (s, 3H)	—	Methyl groups on C-13 and C-17†
	2.17 (s, 3H)	2.07 (s, 3H)	2.17 (s, 3H)	2.22 (s, 3H)	—	
	2.20§ (A_2B_2, 8H)	2.20§ (A_2B_2, 8H)	2.20§ (A_2B_2, 8H)	2.73§ (A_2B_2, 8H)	2.73§ (A_2B_2, 4H)	Ethylene group(s) of propionic acid side chain(s) on C-8 (and C-12)
	–	—	3.43 (s, 6H)	—	—	Carbomethoxy groups of propionic ester side chains on C-8 and C-12
	4.00 (s-broad, 2H)	3.96 (s-broad, 2H)	4.00 (s-broad, 2H)	—	—	Methylene protons on C-10–central methylene bridge
	5.24–7.00 (m, 6H)	—	5.26–6.98 (m, 6H)	5.40/5.43/5.51/5.54/ 6.36/6.39/6.53/6.65/ 6.70/6.84 (m, 3H) 5.67/5.80/5.83/6.73/ 6.86/6.91/7.00/7.08 (m, 3H)	— 5.67/5.80/5.83/6.73/ 6.86/6.91/7.00/7.08 (m, 3H)	Vinyl group on C-18 Vinyl group on C-3
	6.10 (s, 2H)	5.95 (s, 2H)	6.08 (s, 2H)	6.16 (s, 1H)¶ 6.18 (s, 1H)¶	— 6.18 (s, 1H)	Vinyl proton on C-15–methene bridge *c* Vinyl proton on C-5–methene bridge *a*
	–	—	—	7.08–7.67 (m, 10H)	7.08–7.67 (m, 5H)	Aromatic protons of phenylazo group(s)
	9.91 (s-broad, 1H) 10.04 (s-broad, 1H)	9.75 (s-broad, 2H)	9.89 (s-broad, 4/3H)** 10.00 (s-broad, 2/3H)**	10.71 (s-broad, 1H) 10.82 (s-broad, 1H)	— 10.82 (s-broad, 1H)	Amine proton on third ring Amine proton on second ring
	10.45 (s-broad, 1H) 10.49 (s-broad, 1H)	10.31 (s-broad, 2H)	10.51 (s-broad, 1H)** 10.55 (s-broad, 1H)**	11.84 (s-broad, 4H)	11.84 (s-broad, 2H)	Amide proton(s) on first (and fourth) ring†‖
	11.89 (s-broad, 2H)	11.88 (s-broad, 2H)	—			Carboxyl proton(s) of propionic acid side chain(s) on C-8 (and C-12)‖

* $|J|$ 6Hz.

† Signals of the groups cannot be specifically assigned.

‡ Probably two overlapping quartets, $|J|$ 6Hz (4 protons).

§ Centre of A_2B_2 system. The actual signals are located 0.15–0.20p.p.m. upfield and downfield from the centre position, and are partly buried beneath the methyl signals on the one hand and the [$^2H_5$]dimethyl sulphoxide quintet on the other.

‖ Assignment outside parentheses refers to azo pigment $A_{1.1}$.

¶ The definite assignment of the signal at 6.16p.p.m. to the C-15 proton and of the signal at 6.18p.p.m. to the C-5 proton required the measurement of an additional spectrum. This was recorded from a solution of 12.0mg of azo pigment A_1 (V.1+V.2) and 4.5mg of azo pigment $A_{1.1}$ (V.1) in 0.5ml of [$^2H_6$]dimethyl sulphoxide. Thus the molar ratio of azo pigment $A_{1.1}$ to azo pigment $A_{1.2}$ was increased to 1.75:1. The resulting relative enhancement of the signal at 6.18p.p.m. confirmed its assignment to the proton at C-5.

** Integral decreased to approximately half this value after deuterium exchange. The integral resumed its original value on the addition of 1 drop of water to the solution, which was left to stand for 12h at 20°C before spectroscopy.

bilirubin IXβ, IXγ and IXδ (Gray, Nicholson & Nicolaus, 1958), but from neither bilirubin IXα nor bilirubin XIIIα. The occurrence of significant amounts of the last isomer had to be considered since bilirubin compounds isolated from human bile as their phenylazo derivatives and hydrolysed subsequently yielded azo pigments $A_{1.1}$ and $A_{1.2}$ in molar ratios greater than 1:1 (Kuenzle, 1970*b*). This unexpected finding would be compatible with the presence of the symmetrical bilirubin XIIIα, which on azo coupling yields azo pigment $A_{1.1}$ only. However, this possibility was definitely ruled out by the n.m.r. spectrum of human bile bilirubin. The occurrence of bilirubin XIIIα along with bilirubin IXα would have been detected by virtue of increased methyl signals at 1.93 and 2.00p.p.m. as compared to the signals at 2.03 and 2.17p.p.m. However, the integrals of all four methyl signals were found to be identical (Fig. 3*a*), indicating that the sample contained bilirubin IXα only.

The n.m.r. spectra presented in Figs. 3(*a*)–3(*d*) are consistent with the assigned lactam structures (I), (II), (III) and (V), and give no evidence for tautomeric equilibria occurring in dimethyl sulphoxide. In contrast, Nichol & Morell (1969) suggest that bilirubin occurs as the lactim tautomer. Their major argument for a lactim structure stems from the fact that the n.m.r. signal of the carboxyl protons at 11.89p.p.m. does not appear in their spectrum. Consequently they misinterpret the signals of the amide protons occurring at 10.45 and 10.49p.p.m. as arising from the carboxyl protons. The apparent lack of the amide signals thus explains the incorrect conclusion that bilirubin occurs as the lactim. However, a small proportion of bilirubin in solutions of dimethyl sulphoxide and chloroform must be present as the lactim. This is evident from the fact that bilirubin yields some monomethoxy- and dimethoxy-bilirubin dimethyl ester on reacting with diazomethane. Prolonged treatment of bilirubin in dimethyl sulphoxide or chloroform with excess of diazomethane did not enhance the yields of the methoxy derivatives, indicating that esterification either eliminates the reactivity of the lactim hydroxyl or shifts the equilibrium completely in favour of the lactam tautomer. I do not offer a detailed explanation for the mechanism underlying such changes, but suggest that they are possibly the result of some altered interaction between the carboxylic acid groups and the heterocyclic systems of bilirubin. Such an interaction might result from internal hydrogen bonds thought to exist between the carboxylic protons and the pyrrolic rings of bilirubin. [Studies with Dreiding

Table 2. *N.m.r. assignments of bilirubin dimethyl ester in* [^{2}H]*chloroform and carbon tetrachloride* (0.05M *solutions*)

The numbering of carbon atoms refers to the system used for bilirubin (see Fig. 1). Chemical shifts are given in p.p.m. (±0.03) relative to tetramethylsilane = 0 (internal standard). The shape and the relative area (nearest integral value) of each signal are given in parentheses. The following abbreviations are used: s, singlet; m, multiplet; A_2B_2, multiplet of A_2B_2 system.

Solvent ...	Chemical shift (p.p.m.) (shape of signal, relative area)		
	$CDCl_3$	CCl_4	Assignment
	1.75 (s, 1H) 1.96 (s, 2.03 (s, 11H) 2.06 (s, 2.08 (s,	1.55 (s, 1H) 1.66 (s, 2H) 1.96 (s, 2H) 2.02 (s, 2.07 (s, 7H)	Methyl groups on C-2, C-7, C-13 and C-17
	2.67* (A_2B_2, 8H)	2.61* (A_2B_2, 8H)	Ethylene groups of propionic ester side chains on C-8 and C-12
	3.70 (s, 6H)	3.62 (s, 6H)	Carbomethoxy groups of propionic ester side chains on C-8 and C-12
	4.16 (s-broad, 2H)	4.10 (s-broad, 2H)	Methylene protons on C-10 (central methylene bridge)
	4.72–6.70 (m, 8H)	4.68–6.65 (m, 8H)	Vinyl groups on C-3 and C-18 and vinyl protons on C-5 and C-15 (methene bridges *a* and *c*)
	10.08–11.24 (m-broad, 4H)†	10.00–11.16 (m-broad, 4H)	Amine and amide protons on ring nitrogen atoms

* Centre of A_2B_2 system.

† The signals were absent after deuterium exchange, and reappeared on deuterium back-exchange.

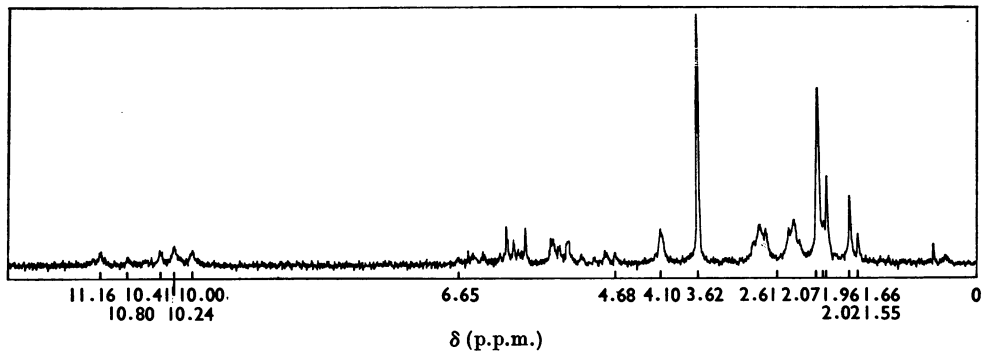


Fig. 4. N.m.r. spectrum of bilirubin dimethyl ester in carbon tetrachloride (0.05M solution). Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). Assignments are given in Table 2. The small signal at far right (0.60 p.p.m.) is a side band of tetramethylsilane. Several molecular species of the compound occur in carbon tetrachloride. This is evident from the presence of five methyl singlets (at 1.55, 1.66, 1.96, 2.02 and 2.07 p.p.m.) and of at least five signals pertaining to the amine and amide protons (at 10.00, 10.24, 10.41, 10.80 and 11.16 p.p.m.).

models suggested that possible hydrogen bonds would point to the centre of the π-orbital system of the pyrrole rings (Musso & von Grunelius, 1959; Eglinton, Martin & Parker, 1965) rather than to the nitrogen atoms (Fog & Jellum, 1963).] Alternatively, such an interaction might be conceived to arise from a betaine structure of bilirubin (von Dobeneck & Brunner, 1965). However, a betaine structure does not seem to occur with bilirubin either in solution or in the crystalline state. This can be deduced from the n.m.r. and i.r. spectra. The evidence for two carboxylic protons and for four protons linked to nitrogen in the n.m.r. spectrum of bilirubin in dimethyl sulphoxide can hardly be assigned to such a structure. Similarly, the i.r. spectrum of crystalline bilirubin is indicative of a carbonylic acid rather than of a carboxylate (carbonyl frequency at 1695 cm^{-1}, which on esterification is shifted to 1740 cm^{-1}), and the same holds for the i.r. spectrum recorded in chloroform (Brodersen, Flodgaard & Krogh-Hansen, 1967).

N.m.r. and optical spectra of bilirubin dimethyl ester revealed the solvent-dependent occurrence of several, probably two, molecular species of this compound. Whereas the n.m.r. spectrum recorded in dimethyl sulphoxide gave evidence of a single molecular species only, the n.m.r. spectrum recorded in [^{2}H]chloroform (inset of Fig. 3c and Table 2) surprisingly showed five methyl signals accounting for a total of 12 protons, and a multiplet of at least five signals was assigned to the four protons on the ring nitrogen atoms. A similar spectrum was also recorded from a solution of the compound in carbon tetrachloride (Fig. 4 and Table 2). The optical spectrum of bilirubin dimethyl ester showed

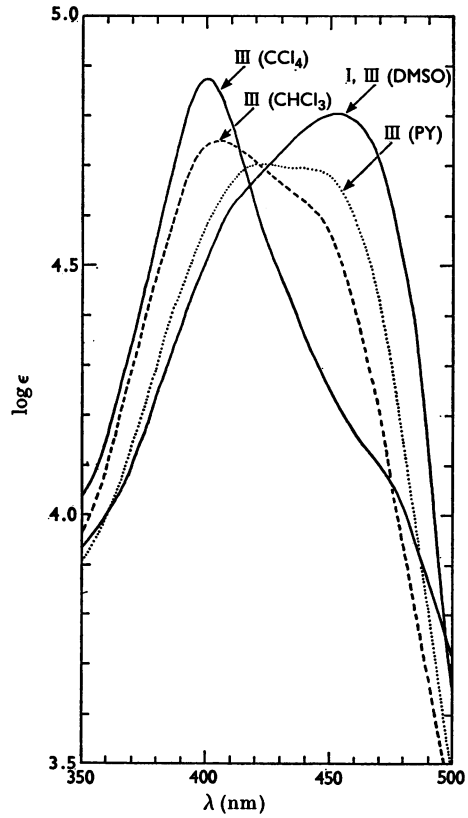


Fig. 5. Optical spectra of bilirubin (I) in dimethyl sulphoxide (DMSO) and of bilirubin dimethyl ester (III) in dimethyl sulphoxide, pyridine (PY), chloroform and carbon tetrachloride. Positions of maxima and molar extinction coefficients (ϵ) are presented in Table 3.

Table 3. *Optical spectra of the model compounds*

For the shapes of the spectral curves see Figs. 5 and 6.

Compound	Solvent	$\lambda_{max.}$ (nm)	ϵ (M^{-1} cm^{-1})
Bilirubin	Dimethyl sulphoxide	453	62200
	Pyridine	450	54300
	$CHCl_3$	453	58800
	CCl_4	453	58900
Mesobilirubin	$CHCl_3$	434	57800
Bilirubin dimethyl ester	Dimethyl sulphoxide	453	63200
	Pyridine	421	50400
		437	49500
	$CHCl_3$	405	55800
		435 (inflexion)	44700
	CCl_4	400	75100
		460 (inflexion)	14100
Dimethoxybilirubin dimethyl ester	Dimethyl sulphoxide	423	—
		450 (inflexion)	—
	$CHCl_3$	418	49900
		445 (inflexion)	40000
	CCl_4	421	—
		446 (inflexion)	—
Azo pigment A_1	Dimethyl sulphoxide–methanolic 0.1 M-acetic acid (1:9, v/v)	270	12700 ± 400*
		277	13200 ± 500*
		327	21200 ± 600*
		514	29600 ± 800*
Azo pigment $A_{1.1}$	Dimethyl sulphoxide–methanolic 0.1 M-acetic acid (1:9, v/v)	270	13000
		277	13100
		327	18200
		514	29100
Azo pigment MESO	Dimethyl sulphoxide–methanolic 0.1 M-acetic acid (1:9, v/v)	270	13000
		277	14800
		315	20400
		506	30100 ± 1200*
Azo pigment ME	Dimethyl sulphoxide–methanolic 0.1 M-acetic acid (1:9, v/v)	270	12600
		277	13400
		327	20800
		514	30000
Azo pigment MME	Dimethyl sulphoxide–methanolic 0.1 M-acetic acid (1:9, v/v)	506	—
		520	—
Azo pigment S_5	Dimethyl sulphoxide–methanolic 0.1 M-acetic acid (1:9, v/v)	272	12000†
		278	12700†
		332	23500†
		520	29100†

* ± 2 S.D., calculated by Student's t test with the use of $\pm t_{0.05}$.

† The computations of the molar extinction coefficients are based on mol.wt. 488.52, and are corrected for 7.3% of inorganic impurity present in the sample.

a strong shift of the maximum to higher wavelengths with increasing polarity of the solvent (Fig. 5 and Table 3). In contrast, the position of the absorption maximum of bilirubin was not influenced by changing the solvent.

The occurrence of several molecular species of bilirubin dimethyl ester can be conceived to result from either one of four possibilities: tautomeric equilibria, *cis–trans* isomerisms, intermolecular aggregates and aggregates between solute and solvent molecules. However, the first three possibilities are not consistent with the experimental data. A significant lactam–lactim tautomerism occurring in carbon tetrachloride is ruled out by the

near-i.r. spectrum, which gave no indication of free or intramolecularly associated hydroxyl groups (no absorption in the range 3663–3425 cm^{-1}). The n.m.r. spectra precluded all other conceivable tautomeric equilibria, i.e. such that involve a proton shift from methyl to vinyl, thus resulting in a cross-conjugated system with methylene and ethylidene groups, proton shifts from either methyl or nitrogen to ring carbon positions and shifts from nitrogen to vinyl end positions. The *cis-trans* isomerisms involving either one or both methene bridges are also ruled out since they are very unlikely to result simply from dissolving the compound in non-polar solvents; and intermolecular aggregates occurring in non-polar solvents are precluded by the optical spectra, which do not show the expected decrease of the molar extinction coefficient with decreasing polarity of the solvent (Table 3).

Aggregates between solute and solvent molecules are thus left as the only possible interpretation, and the following mechanism is proposed to explain the experimental data. von Dobeneck & Brunner (1965) have pointed out that bilirubin in chloroform should theoretically absorb at 420nm. On this basis they have proposed a betaine structure for bilirubin, which would explain the observed absorption at 453nm. It has already been stated above that bilirubin does not form a betaine, but intramolecular hydrogen bonds between the carboxylic protons and the pyrrole rings (see above) would probably have a similar effect on the position of the absorption maximum. Likewise, aggregates with polar solvent molecules might result in such a bathochromic shift. (Dimethyl sulphoxide is known to form strong aggregates with compounds possessing polar groups; in contrast, similar aggregates are rather uncommon with carbon tetrachloride; it is therefore reasonable to assume that bilirubinoids would form strong aggregates with dimethyl sulphoxide rather than with carbon tetrachloride.) This would explain the solvent-dependence of the spectrum of bilirubin dimethyl ester. In non-polar solvents, which cannot form strong aggregates, the compound would absorb at the expected wavelength (420nm). In polar solvents aggregates would be formed between solute and solvent molecules, and these would shift the absorption maximum to higher wavelengths. In contrast, the spectrum of bilirubin would not be solvent dependent since a bathochromic shift would occur not only in polar solvents (by the formation of aggregates with solvent molecules) but also in non-polar solvents (by the formation of intramolecular hydrogen bonds). On the other hand, dimethoxybilirubin dimethyl ester would absorb at the expected wavelength in any solvent as a result of its non-polar character, which would allow neither the formation of aggregates with solvent molecules nor the formation of internal hydrogen bonds.

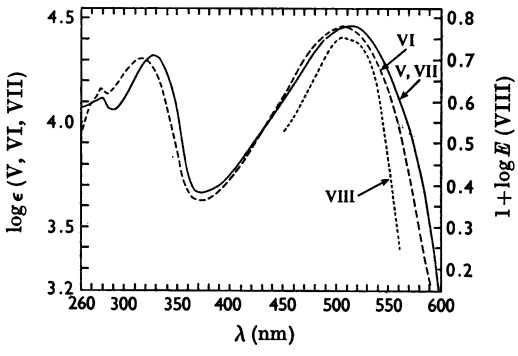


Fig. 6. Optical spectra of azo pigment A_1 (V), azo pigment MESO (VI), azo pigment ME (VII), and azo pigment MME (VIII) in dimethyl sulphoxide–methanolic 0.1M-acetic acid (1:9, v/v). Molar extinction coefficients (ϵ) were calculated for azo pigments A_1, MESO and ME, and are presented in Table 3 together with the exact positions of the maxima. Lack of substance prevented the computation of the ϵ value of azo pigment MME. Therefore an arbitrary scale ($1+\log E$; E is the extinction taken from a qualitative spectrum) was selected to present the spectrum of this compound. It may be noted that the spectra of azo pigment A_1 and azo pigment ME are identical, in agreement with the theoretical prediction. The spectrum of azo pigment $A_{1.1}$ is virtually identical with that of azo pigment A_1, but shows a significantly lower ϵ value at 327nm (see Table 3). The spectrum of azo pigment S_5 (not depicted) shows a slight bathochromic shift as compared with azo pigment A_1 (see Table 3), but otherwise the shapes of both curves are identical.

The occurrence of several molecular species of bilirubin dimethyl ester in non-polar solvents may be explained on this basis. The gradual decrease of the molar extinction coefficient at 420nm and the concomitant increase of the value at 453nm with increasing polarity of the solvent indicate that equilibria exist between solute–solvent aggregates and non-aggregated ester molecules. In dimethyl sulphoxide the equilibrium seems to be shifted completely towards the aggregated form, thus giving rise to only one molecular species. Increasing proportions of non-aggregated molecules appear to be present with decreasing polarity of the solvent. This would explain the occurrence of more than one molecular species of bilirubin dimethyl ester as suggested by the n.m.r. and optical spectra.

Structure of azo pigment S_5. Watson (1958) observed that bilirubin reacts with concentrated sulphuric acid and acetic anhydride to form water-soluble derivatives. The products of this reaction have never been fully identified. Proposed structures comprised sulphates and sulphonates of

bilirubin. Four possible sites of attachment of the sulphur-containing groups were proposed: at the vinyl, carboxyl or lactim hydroxyl groups or at one or more nitrogen atoms of the heterocyclic rings (Gregory & Watson, 1962). In the present investigation the reaction products were converted into their phenylazo derivatives. Reverse-phase partition chromatography revealed the presence of five azo compounds. The most polar of these, termed azo pigment S_5, was estimated to account for approx. 95% of the total azo pigments. The structure of this major compound was elucidated by elemental analysis and by spectroscopic methods. The structure (IX) was assigned to azo pigment S_5 (Fig. 1). Thus the compound consists of both isomers obtained on azo coupling of 3,18-di(ethylidene sulphate)-2,7,13,17-tetramethylbiladiene-*ac*-8,12-dipropionic acid (for the numbering system used see the legend to Fig. 1).

Elemental analysis revealed the presence of 7.3% of inorganic material, probably silica. When the results of the elemental analysis were corrected for this impurity, the values obtained were in agreement with the assigned structure [Found: C, 55.3; H, 5.2; N, 11.6; S, 6.8. Calc. for $C_{22}H_{24}N_4O_7S$ (mol.wt. 488.52): C, 54.1; H, 4.9; N, 11.5; S, 6.6%].

The n.m.r. spectrum of azo pigment S_5 is shown in Fig. 7. The following assignments were made in agreement with the proposed structure (IX). The group of signals at 1.16 and 1.20p.p.m. was interpreted as consisting of two overlapping triplets (coupling constant, $|J|$ approx. 8Hz, integral corresponding to 6 protons). These were assigned to either one of the two methyl groups of the ethylidene sulphate side chains. The singlets at 2.13p.p.m. (6 protons) and 2.23p.p.m. (6 protons) were assigned to the four methyl groups at ring positions C-2, C-7, C-13 and C-17. The multiplet of an A_2B_2 system (8 protons) was found to be centred at 2.73p.p.m. with its high-field portion being almost completely buried in the [$^2H_5$]dimethyl sulphoxide quintet at 2.49p.p.m. This multiplet was assigned to the ethylene groups of the propionic acid side chains. An unusually broad water signal was positioned at 3.58p.p.m. instead of at 3.33p.p.m. This shift was attributed to a proton exchange involving the acid proton of the sulphate group. The singlet at 6.21p.p.m. (2 protons) was assigned to the vinyl protons of the methene bridges (*a*) and (*c*). The group of broad signals at 6.56–6.86p.p.m. (4 protons) was assigned to the amine and amide protons of the pyrrole and the α-pyrrolone rings respectively. The multitude of signals at 7.14–7.70p.p.m. (10 protons) accounted for the aromatic protons of both phenylazo groups. The broad signal at 10.74p.p.m. (2 protons) was assigned to the carboxyl groups of the propionic acid side chains. The methine protons of both ethylidene sulphate side chains were not detected. They were probably buried beneath the [$^2H_5$]dimethyl sulphoxide quintet at 2.49p.p.m. The n.m.r. spectrum is not compatible with any other than the assigned structure (IX). It certainly precludes the presence of a sulphonic acid group. 'Aromatic' sulphonation would be conceivable either at the methene bridges or at the vinyl side chains. Both possibilities are ruled out by the evidence for the protons at the methene bridges and by the absence of further vinyl signals respectively. Thus the sulphur-containing group must be a sulphate radical introduced at the vinyl side chains by the addition of sulphuric acid according to Markownikoff's rule.

Further evidence for the assigned structure was obtained from the i.r. spectrum of azo pigment S_5 (Fig. 8). The broad bands at approx. 3350 and 3150cm^{-1} were assigned to the N–H, CO·O–H and O–H (of O·SO_3H) stretching modes. The rather broad absorption at 1704cm^{-1} was interpreted as pertaining to both the C=O stretching frequencies of the carboxyl and α-pyrrolone groups. The broad bands at 1235, 1038 and 833cm^{-1} were assigned to the stretching modes of the sulphate group, in accordance with the i.r. data presented by Lloyd, Tudball & Dodgson (1961) for *O*-sulphates of

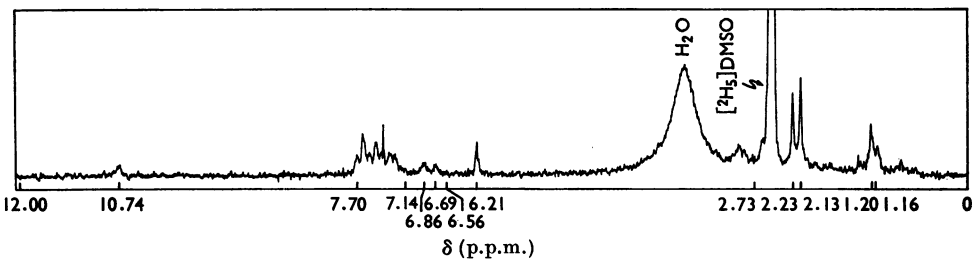


Fig. 7. N.m.r. spectrum of azo pigment S_5 in [$^2H_6$]dimethyl sulphoxide ([$^2H_6$]DMSO). The spectrum was recorded from a saturated solution (approx. 0.01M). Chemical shifts are given in p.p.m. relative to tetramethylsilane = 0 (internal standard). Assignments are presented in the text.

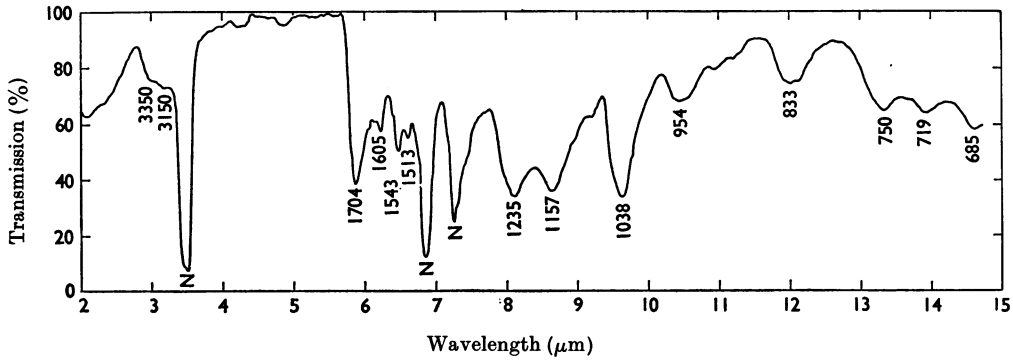


Fig. 8. I.r. spectrum of azo pigment S_5 (Nujol mull). Positions of bands are given in cm^{-1}. Nujol absorptions are marked with N. Assignments are presented in the text.

various compounds. The assignments were as follows: S–O vibration at 1235 cm^{-1}, C–O (of C–O–S) at 1038 cm^{-1} and O–S (of C–O–S) at 833 cm^{-1}. Thus a sulphonic acid group is ruled out (Schreiber, 1949). Further, the C–O–S vibrations preclude attachment of the sulphate group at the nitrogen atoms of the rings; but they would be compatible with a linkage either at the carboxyl or the lactim hydroxyl function. Attachment at the carboxyl group is ruled out by the absence of vibrations typical of anhydrides (in the range 1825–1725 cm^{-1}). On the other hand, an *O*-sulphate involving the lactim hydroxyl group is not definitely precluded by the i.r. spectrum, since the absence of the amide band I would not be detected due to the presence of the carboxyl C=O vibration in this region.

The optical spectrum of azo pigment S_5 showed a slight bathochromic shift as compared with the spectrum of azo pigment A_1, but otherwise the shapes of both curves were identical (Fig. 6 and Table 3). There was a single maximum at 520 nm, unlike the double absorption observed with azo pigment MME (Fig. 6). This I believe to rule against attachment of the sulphate radical at the lactim hydroxyl group. The bathochromic shift, as compared with the spectrum of azo pigment A_1, is an unexplained finding, with a hypsochromic shift having been expected. However, I consider that this feature is not sufficient evidence against the assigned structure (IX), particularly when weighed against the supporting n.m.r. and i.r. data.

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