Validation of infrared spectroscopy for assessment of vinyl polymers of bile-pigment gallstones

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The i.r. spectra of bilirubin isomers that differ in number and position of vinyl groups were examined to verify the assignment of the 988 cm^{-1} peak of bilirubin (991 cm^{-1} peak in calcium bilirubinate) to its pendant vinyl groups. There were only small changes in this peak with changes in position of vinyl groups (exo-2- and -18-vinyl versus endo-3- and -17-vinyl), but progressive loss of peaks in this region was observed when vinyl groups were reduced to ethyl groups (dihydrobilirubin and mesobilirubin). Methylvinylmaleimide, a monopyrrole derived from the outer (A and D) rings of bilirubin, has a pendant vinyl group and exhibits a prominent peak at 986 cm^{-1} , but haematinic acid methyl ester derived from the inner (B and C) rings has no vinyl group and shows no peak near 988 cm^{-1} . These observations verify the assignment of the 988 cm^{-1} peak of bilirubin to its pendant vinyl groups. This supports our previous proposal that a decrease in the peak at $985\text{--}995\text{ cm}^{-1}$ in the i.r. spectra of pigment gallstones, as compared with unconjugated bilirubin or calcium bilirubinate, indicates a consumption of vinyl groups in the process of formation of the polymer in the pigment stones.

The close similarity of the i.r. spectra of unconjugated bilirubin and calcium bilirubinate with the i.r. spectra of pigment gallstones has been established (Suzuki, 1965a,b; Suzuki & Toyoda, 1966). Quantification by i.r. spectroscopy of the components of human gallstones has been verified in 25 pigment and 31 cholesterol gallstones (Trotman *et al.*, 1977). Despite the similarities, differences exist between the i.r. spectra of unconjugated bilirubin and calcium bilirubinate, as compared with pigment gallstones. We had consistently noticed markedly decreased absorbance between 985 and 995 cm^{-1} in pigment stones, especially of the black type, as compared with unconjugated bilirubin and calcium bilirubinate (Black *et al.*, 1982; Ohkubo *et al.*, 1984). On a theoretical basis, these peaks were assigned to the

pendant vinyl groups of unconjugated bilirubin (988 cm^{-1}) and calcium bilirubinate (991 cm^{-1}) (Suzuki & Toyoda, 1966).

Polymer chemists have often evaluated the extent of vinyl polymerization or of vinyl group reduction from the loss of i.r. peaks in this same region (Hummel, 1974; Rodriguez, 1970). We therefore proposed that the decrease in the $985\text{--}995\text{ cm}^{-1}$ peaks in pigment stones, as compared with unconjugated bilirubin or calcium bilirubinate, represented consumption of the vinyl groups in the process of polymer formation in pigment stones (Ohkubo *et al.*, 1984).

To assess accurately the significance of the loss of the $985\text{--}995\text{ cm}^{-1}$ peak in pigment stones, we considered that the assignment of these peaks needed to be validated for bile pigments and other pyrroles. We therefore studied the i.r. spectra of biladienes-(a,c) that differed in number and position of vinyl groups, and of monopyrroles derived from bilirubin's outer (A and D) rings, which have vinyl groups, and the inner (B and C) rings, which lack vinyl groups.

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Materials and methods

Preparation of bilirubin isomers and derivatives

The III α , IX α and XIII α isomers of unconjugated bilirubin (Fig. 1) were prepared by the acid-mediated dipyrrole-exchange method (McDonagh & Assisi, 1972). Mesobilirubin IX α (Fig. 1) was prepared by reduction of unconjugated bilirubin as follows. Unconjugated bilirubin (Gallard-Schlesinger, Carle Place, NY, U.S.A.), 10mg in methanol (10ml) containing 50mg of ascorbic acid and a trace of EDTA, was alkalized with 1M-NaOH (0.5ml/mg of unconjugated bilirubin) and was shaken for 3 min with Pd/charcoal catalyst at room temperature under H₂ at a pressure of 80kPa (0.8atm) (Blanckaert *et al.*, 1976). Dihydrobilirubin (Fig. 1) was prepared by keeping 10mg of unconjugated bilirubin with 15mg of Pd/charcoal catalyst in 6ml of distilled chloroform for 12h at 41°C under H₂ at a pressure of 4MPa (40atm) (Fischer, 1914; Fischer & Niemann, 1924; Pearson & Watson, 1963; Kay *et al.*, 1963). After purification by two stage t.l.c. on silica gel G (Analtech, Newark, DE, U.S.A.) developed in chloroform/acetic acid (100:1, then 500:1, v/v) (McDonagh & Assisi, 1972), the bilirubin isomers were eluted with chloroform.

The monopyrroles methylvinylmaleimide and haematinic acid (Fig. 2) were prepared by controlled chromic acid degradation on unconjugated bilirubin (Rudiger, 1969, 1970). Products were

extracted into diethyl ether and separated by t.l.c. on silica gel G developed in carbon tetrachloride/cyclohexane/ethyl acetate (5:3:1, by vol.) (Rudiger, 1969). Methylvinylmaleimide was identified by its characteristic fluorescence with u.v. light at 254nm and was purified by sublimation at 64°C at a vacuum of 0.7 Pa (0.005mmHg) (Bonnett & McDonagh, 1969). Haematinic acid was identified with chlorine/benzidine and dinitrophenylhydrazine (Rudiger, 1969), eluted from the gel with chloroform, and methylated with 5% BF₃ as described below for the methylation of azopigments. Haematinic acid methyl ester was purified by t.l.c. on silica gel G developed in ethyl acetate (Lightner & Quistad, 1972).

Verification of structures of the bilirubin derivatives

Tetrapyrroles were characterized by their typical visible-region spectral peaks (Table 1) and by the pattern of methylated azopigments formed (Table 2). To prepare azopigments, about 100 μ g of each tetrapyrrole was dissolved in 10ml of dimethyl sulphoxide plus 0.1ml of chloroform, and was treated twice with 1.0ml of ethyl anthranilate diazo reagent for 15min (Blanckaert *et al.*, 1976). After addition of 1.0ml of ascorbic acid (10mg/ml of water), azopigments were extracted into chloroform, and washed successively with 5ml of glycine/HCl buffer, pH 2.7, 20ml of 10% (w/v) NaCl solution and 5ml of deionized water. After water was removed by filtration through chloroform-saturated Whatman no. 1 filter paper, the chloroform was removed by warming at 45°C under a stream of N₂. The residue was redissolved in 1.0ml of pentan-2-one and 1.0ml of dry methanol, and the azopigments were then methylated by refluxing at 66°C with 5% (w/v) BF₃ (final concentration) in dry methanol. Methylated azopigments were extracted into chloroform, washed with deionized water and separated by two-stage t.l.c. on silica gel G, developed in chloroform/

Fig. 1. Structures of (a) bilirubin IX α , (b) bilirubin XIII α , (c) bilirubin III α , (d) dihydrobilirubin and (e) mesobilirubin IX α .

Fig. 2. Structures of (a) methylvinylmaleimide and (b) haematinic acid.

Table 1. Influence of position and number of vinyl groups on absorbance peaks of bilirubin classically assigned to vinyl groups

Compounds	Vinyl groups*		Visible-region-spectrum $\lambda_{\text{max.}}$ (CHCl ₃) (nm)	I.r.-spectrum A_{peak}/A_{1614} ratio (KBr discs)		
	Ring A	Ring D		I.r. peak ... 988cm	932cm	918cm
Bilirubin III α	Exo-2-	Exo-18-	455	0.250	0.129	0.071
Bilirubin IX α	Endo-3-	Exo-18-	453	0.259	0.111	0.104
Bilirubin XIII α	Endo-3-	Endo-17-	448	0.275	0.204	0.134
Dihydrobilirubin	Endo-3-	0 (exo-18-ethyl)	440	0.093†	0.102	0
Mesobilirubin	0 (endo-3-ethyl)	0 (exo-18-ethyl)	433	0‡	0.200	0

* The number refers to the ring carbon from which the vinyl or ethyl group is pendant (Fig. 1).

† Also peak at $1005\text{cm}^{-1}=0.021$ and $964\text{cm}^{-1}=0.036$.

‡ No 988cm^{-1} peak, but has $1004\text{cm}^{-1}=0.086$ and $978\text{cm}^{-1}=0.059$.

Table 2. Patterns on t.l.c. obtained with methylated azopigments derived from the tetrapyrroles

T.l.c. was in silica gel G developed with chloroform/methanol (9:1, v/v) for 1cm followed by benzene/ethyl acetate (9:1, v/v) for 15cm.

Compound	Azopigments*	R_F
Bilirubin III α	Isovinyl	0.547
Bilirubin IX α	Isovinyl	0.547
	Vinyl	0.433
Bilirubin XIII α	Vinyl	0.433
Dihydrobilirubin	Vinyl	0.433
	'Isoethyl'	0.393
Mesobilirubin IX α	'Isoethyl'	0.453
	'Ethyl'	0.393

* Vinyl azobilirubin arises from splitting of the central methylene bridge of a bilidien with vinyl groups on the 2- and/or 18-carbon atoms (Fig. 1). Isovinyl azobilirubin, likewise, arises from bilidienes with vinyl groups on the 3- and/or 17-carbon atoms (Fig. 1) (Jansen & Stoll, 1971).

methanol (9:1, v/v) for 1cm followed by benzene/ethyl acetate (9:1, v/v) for 15cm (Blanckaert *et al.*, 1976). The expected azopigment patterns (Blanckaert *et al.*, 1976) were obtained (Table 2).

Structures of mesobilirubin and dihydrobilirubin were further verified by proton-n.m.r. spectroscopy in a JEOL (Crawford, NJ, U.S.A.) FX 270 Super Conducting NMR Spectrometer: the spectrum of mesobilirubin included the resonances at δ (p.p.m.) (C^2HCl_3) 1.03–1.13 (6H, m, CH_3), 2.35 (2H, q, CH_2) and 2.51 (2H, q, CH_2) corresponding to the ethyl groups on the A and D rings (Kaplan & Navon, 1981), and the spectrum of dihydrobilirubin included the resonances at δ (p.p.m.) (C^2HCl_3) 1.03–1.13 (6H, m, CH_3) and 2.35 (2H, q, CH_2) plus the resonances corresponding to the characteristic ABX signals of the endo-3-vinyl position (δ_A , δ_B , $\delta_X = 5.60$, 5.59, 6.61 and J_{AB} , J_{AX} , $J_{BX} = 1.9$, 11.2, 17.8) (Kaplan & Navon,

1981). The structures of methylvinylmaleimide, haematic acid methyl ester and dihydrobilirubin were further verified by mass spectroscopy (5985A-GCG-MS Electron Impact Ionization; Hewlett-Packard, Palo Alto, CA, U.S.A.): methylvinylmaleimide m/z 137 (36%, M^+), 119 (5%, $M^+ - \text{H}_2\text{O}$), 109 (17%, $M^+ - \text{CO}$), 94 (7%, $M^+ - \text{NHCO}$), 66 (100%, $M^+ - \text{CONHCO}$) and 65 (38%, $M^+ - \text{H} - \text{CONHCO}$); haematic acid methyl ester m/z 197 (2%, M^+), 166 (34%, $M^+ - \text{OCH}_3$), 165 (100%, $M^+ - \text{H} - \text{OCH}_3$) and 137 (71%, $M^+ - \text{CO}_2\text{CH}_3$) (Jackson *et al.*, 1967). Dihydrobilirubin demonstrated the ions at m/z 586 (6%, M^+) and at m/z 301, 299, 288 and 286, which correspond to fragmentations on either side of the central methylene groups (Fig. 1) (Jackson *et al.*, 1967). Methylvinylmaleimide also exhibited a characteristic u.v. spectral peak in methanol at 227nm (Bonnett & McDonagh, 1969).

I.r. spectroscopy

Approx. 0.4mg of desiccated pigment was jointly ground in an agate mortar and pestle with $100 \pm 0.02\text{mg}$ of desiccated i.r.-grade KBr (Gallard-Schlesinger) and compressed in an evacuated stainless-steel die at 15MPa (22000lbf/in^2) for 12min. The wafer was immediately subjected to i.r. spectroscopy in a Perkin-Elmer (Norwalk, CT, U.S.A.) model 727 instrument. Dihydrobilirubin, which could be produced in only small amounts, was subjected to i.r. spectroscopy on a more sensitive Nicolet (Madison, WI, U.S.A.) 7199 FTIR instrument. The magnitude of absorbance peaks was corrected for the amount of pigment present in each wafer by dividing by the absorbance at 1614cm^{-1} , which is proportional to the amount of polypyrrole present (Trotman *et al.*, 1977). [The reference peak for the amount of unconjugated bilirubin is 1614cm^{-1} , but this shifts to 1624cm^{-1} for calcium bilirubinate (Trotman *et al.*, 1977).]

Results

I.r. spectra of bilirubin isomers that differed in the exo-2- and -18- versus endo-3- and -17-positions of vinyl groups exhibited only small differences in the intensity of the peak near

Fig. 3. I.r. spectra of (a) bilirubin III α , (b) bilirubin IX α and (c) bilirubin XIII α .

990 cm^{-1} , despite larger differences in the vicinity of 918–932 cm^{-1} . There was a tendency for the intensity of these last peaks to increase with endo-3- and -17-vinyl as compared with exo-2- and -18-vinyl groups, but no exact quantitative relationship was found (Fig. 3 and Table 1). A marked displacement and diminution of peaks near 990 cm^{-1} occurred as vinyl groups were reduced to ethyl groups in dihydrobilirubin and mesobilirubin (Fig. 4 and Table 1).

Methylvinylmaleimide, which is a monopyrrole derived from the A or D ring of bilirubin and has a pendant vinyl group, demonstrated a prominent peak at 986 cm^{-1} (Fig. 5). On the other hand, haematinic acid methyl ester, which is derived from the B or C ring of bilirubin and lacks pendant vinyl groups but has a pendant propionic acid group, shows little absorbance in the 990 cm^{-1} region (Fig. 5).

Discussion

I.r. spectroscopy is a valid method for evaluation of the composition of gallstones (Suzuki, 1965*a,b*; Suzuki & Toyoda, 1966; Trotman *et al.*, 1977). We had observed that the 988 cm^{-1} and 991 cm^{-1} peaks of bilirubin and calcium bilirubinate respectively are markedly diminished in pigment gall-

Fig. 4. I.r. spectra of (a) mesobilirubin IX α and (b) dihydrobilirubin.

Fig. 5. I.r. spectra of (a) polymethylvinylmaleimide, (b) methylvinylmaleimide and (c) haematinic acid

stones, which are thought to be largely composed of either calcium bilirubinate or a derivative thereof (Suzuki, 1965b; Suzuki & Toyoda, 1966; Wosiewicz, 1977; Wosiewicz & Schroebler, 1977, 1978). We had concluded that the loss of the 991 cm⁻¹ i.r. peak represented loss of vinyl groups, on the theoretical basis of an assignment of these peaks to the pendant vinyl groups of bilirubin

(Suzuki & Toyoda, 1966). In fact, i.r. peaks between 905 and 925 cm⁻¹ and between 980 and 995 cm⁻¹ are characteristically assigned to the C-H out-of-plane bending of linear olefins of the type R-CH=CH₂ (Rodriguez, 1970). In addition, peaks in this same region have been used to assess the degree of vinyl polymerization in commercial polymers (Rodriguez, 1970). The differences in this region are exemplified in the i.r. spectra of styrene and its polymer polystyrene and of vinylpyrrolidone and its polymer polyvinylpyrrolidone (Pouchert, 1981). Polymerization of methylvinylmaleimide as described by Lightner & Quistad (1971) is associated also with marked diminution of the i.r. peak at 986 cm⁻¹ found in this compound (Fig. 3).

Review of published spectra of pyrrole rings with pendant side chains revealed that compounds with pendant vinyl groups had absorption peaks between 950 and 1000 cm⁻¹. Compounds with unsaturated rings had absorptions (Nujol) near 990 cm⁻¹ as exemplified by 1-vinyl-2-pyrrolidone (985 cm⁻¹) (Sadler, 1978) and styrene (990 cm⁻¹) (Pouchert, 1981). Compounds without vinyl groups had no peaks between 950 and 1000 cm⁻¹ (maleimide, *N*-methylmaleimide, pyrrole, pyrrole-2-carboxylic acid, pyrrole-2-carboxaldehyde, polyvinylpyrrolidone, succinimide, *N*-methylsuccinimide and polystyrene) with the exception of 2,5-dimethylpyrrole (995 cm⁻¹), 1,2,5-trimethylpyrrole (995 and 970 cm⁻¹) and 3-ethyl-2,4-dimethylpyrrole (980 and 950 cm⁻¹) (Pouchert, 1981). The peaks between 980 and 995 cm⁻¹ in these three monopyrroles could confuse the quantification of pyrrole vinyl groups when the 991 cm⁻¹ i.r. peak is used, but these derivatives of bilirubin are unlikely to be found in gallstones, and their presence would increase, not decrease, the 991 cm⁻¹ peak in these stones.

Our findings are consistent with the i.r. absorptions found for vinyl groups on pyrrole rings as summarized above, and this work directly confirms the assignment of the 988 cm⁻¹ peak of unconjugated bilirubin (991 cm⁻¹ peak of calcium bilirubinate) on i.r. spectrum to its pendant vinyl groups. A peak in this vicinity was prominent in methylvinylmaleimide, which has a vinyl group, but was barely detectable in haematinic acid methyl ester, which does not. The peak also diminished on reduction of the vinyl groups to ethyl groups, as occurred in the formation of dihydrobilirubin and mesobilirubin, or as would occur during vinyl polymerization of bilirubin. The similarity in the intensity and location of the 988 cm⁻¹ peak among bilirubin IXα and its IIIα and XIIIα isomers indicates that quantification of vinyl groups from this peak is influenced little by the endo-3- and -17- versus exo-2- and -18-

positions of the vinyl groups on the A and D rings (Fig. 1). By contrast, peaks at 918 and 932 cm⁻¹ were altered by such positional shifts in vinyl groups, indicating that they are not reliable for the monitoring of vinyl groups in bilirubin.

Validation of the assignment of the 988 cm⁻¹ peak to the pendant vinyl groups in unconjugated bilirubin (991 cm⁻¹ peak of calcium bilirubinate) allows these i.r. peaks to be used to assess vinyl groups in bilirubin and related compounds. This includes pigment gallstones, since these stones have been shown to contain calcium bilirubinate and an insoluble residue thought to be formed by polymerization of bilirubin or a bilirubin-like substance (Suzuki, 1965*b*; Suzuki & Toyoda, 1966; Wosiewicz, 1977; Wosiewicz & Schroebl, 1977, 1978). Studies in our laboratory have shown a correlation of the relative loss of absorbance at 991 cm⁻¹ (with absorbance at 1614 cm⁻¹ as a reference peak for the amount of polypyrrole present) with the amount of polymer in the stone, as assessed by either the amount of insoluble residue present or by the equilibrium swelling of the stone (Black *et al.*, 1982; Ohkubo *et al.*, 1984). These data indicate that vinyl groups are consumed during the polymerization process, most probably as a result of their direct involvement in the polymerization process.

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