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Synthesis and Separation by Thin-Layer Chromatography of Bilirubin-IX Isomers

THEIR IDENTIFICATION AS TETRAPYRROLES AND DIPYRRROLIC ETHYL ANTHRANILATE AZO DERIVATIVES

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Procedures for the synthesis, separation and determination of structure of the bilirubin-IX isomers are described. 1. The four biliverdin-IX isomers were prepared by oxidative cleavage of haemin and were separated as their dimethyl esters. The individual esters were reduced with $NaBH_4$, and the bilirubin esters obtained were subjected to alkaline hydrolysis yielding the corresponding bilirubin-IX isomers. 2. The bilirubin-IX isomers were structurally characterized (*a*) at the tetrapyrrolic stage by mass spectrometry of their trimethylsilyl derivatives and (*b*) by formation and structural analysis of their dipyrrolic ethyl anthranilate azo derivatives. 3. The absorption spectrum of bilirubin-IXα differed strikingly from the spectra of the other isomers. The presence of a pronounced shoulder around 453 nm in the spectrum of bilirubin-IXβ allows easy differentiation from bilirubin-IXδ. Methylation of the carboxyl groups largely eliminates the spectral differences between the IXα- and non-α isomers. 4. The bilirubin-IX isomers are conveniently separated by t.l.c. Detection and unequivocal identification is possible on a micro-scale by (*a*) t.l.c. with respect to reference compounds and (*b*) subsequent formation and t.l.c. of the more stable ethyl anthranilate azopigments. 5. Pronounced differences in polarity, i.e. solvent distribution, between the bilirubin-IX isomers indicate that a re-evaluation of conclusions reached previously with regard to the presence in, or absence from, biological fluids of some isomers and their relative amounts is needed.

A major hindrance to solving problems related to haem catabolism resides in technical difficulties in determining the isomeric composition of biliverdin and bilirubin pigments (Gray *et al.*, 1972). A useful approach to this problem consists of analysis of monopyrrolic oxidative-degradation products (Nicolaus, 1960), especially with the more sensitive modifications of Rüdiger (1969) and Tipton & Gray (1971), but, in general, to reach definite conclusions separation of the tetrapyrroles before analysis is required. Also, the procedures suffer from low yields of some reaction products.

Three t.l.c. systems for separation and determination of bile pigments as the biliverdin dimethyl esters have been described (Rüdiger, 1969; O'Carra & Colleran, 1970; Bonnett & McDonagh, 1973). However, when the verdin derivatives are obtained by oxidation of bile pigments containing a central methylene bridge, possible formation of other tetrapyrroles, due to dipyrrole exchange (Bonnett & McDonagh, 1970; Stoll & Gray, 1970), has to be taken into account. Therefore direct separation without previous modification would be preferable. Further, as in general, relatively large losses of some bilirubin-IX isomers can occur owing to their different polarities (the present work) a re-evaluation of the importance of non-α isomers is required.

T.l.c. of the IIIα, IXα and XIIIα isomers of bilirubin has been reported (McDonagh & Assisi, 1971). In the present work, systems for t.l.c. of bilirubin-IXα, -IXβ, -IXγ and -IXδ and of the tetrapyrroles obtained from each by dipyrrole exchange have been developed and validated. The reference compounds were synthesized and structurally characterized. On a micro-scale, elucidation of separated bilirubins is done conveniently by reference to known isomers and by analysis of their ethyl anthranilate azo derivatives.

Materials and Methods

Chemicals

The chloroform contained 0.6% ethanol as a stabilizer. Pentan-2-one (previously dried on $CaSO_4$) was redistilled before use. Aqueous solutions were prepared with twice-glass-distilled water. Haemin was from Koch–Light Laboratories (Colnbrook, Bucks., U.K.), BF_3 from Aldrich-Europe (Janssen Pharmaceutica, Beerse, Belgium) and *NN*-dimethylformamide (Uvasol) from Merck A.G. (Darmstadt,

Germany), $NaBH_4$ and 10% (w/w) Pd adsorbed on $CaCO_3$ were from Fluka A.G. (Buchs, Switzerland). All other reagents were of analytical reagent grade.

Methods

Except when stated otherwise chromatography was done at room temperature (20°C) in the dark on glass plates pre-coated with silica gel (DC-Kieselgel F254, 5715/0025; Merck A.G.). For separation of bile pigments the tanks were flushed with N_2 before the chromatographic developments were started. All centrifugations were done at room temperature in an ordinary laboratory centrifuge at 3000rev./min ($1000g_{av.}$). The glycine/HCl buffer was prepared at room temperature by adjusting 0.4M-HCl to pH2.7 with solid glycine.

Formation of biliverdin dimethyl esters from haemin and separation by t.l.c. Verdohaemochrome formation from haemin/oxygen/ascorbic acid mixtures, and subsequent preparation of biliverdin dimethyl esters were done by the procedure of Bonnett & McDonagh (1973). For preparative t.l.c. of the esters it was slightly modified as follows. Pigments were chromatographed on pre-coated silica-gel plates by development with chloroform/acetone (19:1, v/v). Separation of the IXα and IXγ isomers was improved by opening the tanks when the solvent front had reached the upper edge of the plates. When the plates were visibly dry to a depth of 5cm from the upper edge the tanks were closed again and t.l.c. was continued. Occasional inspection of the plates during chromatography indicated whether it was useful or not to repeat the drying process. For optimal separation care should be taken not to overload the plates.

Analytical t.l.c. of separated verdin esters was done by parallel analyses by either the procedure described above, or the method of O'Carra & Colleran (1970).

Formation of bilirubin dimethyl esters from biliverdin dimethyl esters by reduction with $NaBH_4$. Bands of biliverdin dimethyl esters were scraped from thin-layer plates, and the silica-gel powders mixed with methanol. To each verdin solution (concn. about 20μM) small amounts of powdered $NaBH_4$ were added at room temperature, with continuous stirring, until a clear yellow colour was obtained (duration of treatment, 3–5min). The solution was placed on ice, mixed with 1vol. of chloroform and further acidified with 4vol. of ice-cold glycine/HCl buffer, pH2.7, containing ascorbic acid (10mg/ml). The chloroform mixture was washed three times with 4vol. of distilled water, dried by filtration through chloroform-moistened filter paper and finally concentrated *in vacuo* under N_2 at 30°C in a rotary evaporator. Excessive evaporation was avoided because otherwise complete solution in methanol, which is needed for subsequent treatment, was difficult. During all treatments the bile pigment solutions were briskly flushed with N_2 and were kept on ice.

Alkaline hydrolysis of bilirubin dimethyl esters. Bilirubin dimethyl ester (2μmol) was dissolved in 10ml of methanol, containing 50mg of ascorbic acid and a trace of EDTA (disodium salt). The mixture was treated with 5ml of 1M-NaOH (final pH13–13.5) and shaken for 30min in the dark at 37°C in a closed vessel provided with a N_2 atmosphere. After acidification with 625μl of acetic acid (final pH about 5.5) 10ml of chloroform and 20ml of glycine/HCl buffer, pH2.7, saturated with $(NH_4)_2SO_4$, were added. After thorough shaking, the organic phase was collected, and the aqueous phase extracted further with 5ml of chloroform. The combined extracts were filtered on chloroform-moistened filter paper.

Separation of the isomers of bilirubin-IX. For analytical t.l.c., solutions of bilirubin-IX in chloroform were applied to thin-layer plates, which were developed over 18cm with chloroform/methanol/water (40:9:1, by vol.). Preparative t.l.c. was initiated with the same solvent mixture. Bilirubin-IXα was eluted with chloroform and rechromatographed with chloroform/methanol (50:1, v/v). Methanol eluates of the IXβ, IXγ and IXδ isomers were concentrated *in vacuo* under N_2 and further diluted with an equal volume of chloroform. The pigments were rechromatographed with chloroform/methanol (4:1, v/v) on thin-layer plates, provided at the upper edge with a pad of filter paper.

Preparation of mesobilirubin-IX by hydrogenation. The procedure is based on work of Gray *et al.* (1961). Methanol solutions (10ml) of purified bilirubin-IX isomers (about 5μmol) were mixed with 5ml of 1M-NaOH containing 50mg of ascorbic acid and a trace of EDTA. The mixtures were provided with about 50mg of Pd catalyst and were shaken for 3min at room temperature at a H_2 pressure of 81kPa (0.8atm). After centrifugation, the supernatants were decanted and set aside and the sediments were washed with 10ml of methanol. The combined bile pigment solutions were acidified with 625μl of acetic acid and were further extracted as indicated under 'Alkaline hydrolysis of bilirubin dimethyl esters'.

Formation and analysis of ethyl anthranilate azopigments. Bands or spots of separated bilirubin-IX isomers and of meso derivatives were scraped from thin-layer plates and the powders transferred to 10ml centrifuge tubes. To ensure complete reaction with diazotized ethyl anthranilate of slowly reacting substances, such as bilirubin-IXα, the procedure of Heirwegh *et al.* (1975) was modified as follows. The silica-gel powders were mixed with 1vol. of diazo reagent, 2vol. of formamide and 2vol. of ethanol. After reaction at room temperature for 3–5min, with intermittent mixing about every 30s, 3vol. of pentanone and 5vol. of glycine/HCl buffer, pH2.7, were added, and the tubes shaken vigorously. After centrifugation the organic phases were washed exhaustively with glycine/HCl buffer, pH2.7, to

remove formamide. The extracts were applied to thin-layer plates and submitted to the following development sequence: (*a*) chloroform/methanol/water (65:25:3, by vol.) (over 2cm), (*b*) benzene/ethyl acetate (17:3, v/v) (over 18cm), and (*c*) chloroform/methanol/water (65:25:3, by vol.) (over 18cm) and/or chloroform/methanol (9:1, v/v) (over 15cm).

For preparative work 1 vol. of purified preparations of bilirubin-IX isomer in chloroform (α isomer) or chloroform/methanol (1:1, v/v) (β, γ and δ isomers) was treated with 8vol. of propan-1-ol. After the addition of ethyl anthranilate diazo reagent, preparation and extraction of the azo derivatives were done by the procedure of Van Roy & Heirwegh (1968). The azopigments were purified by t.l.c. by using the development sequence given above. Azopigments IID and IVG (see Scheme 2) and their meso derivatives were further purified by t.l.c. with benzene/ethyl acetate (17:3, v/v) followed by elution with chloroform and rechromatography with benzene/hexane (1:1, v/v).

Methyl esters of the azopigments were prepared as follows. Azopigments purified by t.l.c. were eluted from the silica-gel powders with pentanone/formamide (9:1, v/v). After the eluates had been washed three times with 4vol. of glycine/HCl buffer, pH2.7, the organic phases were treated with an equal volume of ethereal diazomethane. After 15–30s the excess of reagent was evaporated, and the organic solutions were applied to thin-layer plates. T.l.c. of the methyl esters of azopigments IA, IB, IIIE and IIIF and of the meso derivatives was done with benzene/ethyl acetate (9:1, v/v), the esters of azopigments IIC and IVC being developed with benzene/ethyl acetate (17:3, v/v). Reference preparations of azopigments IA and IB and of their methyl esters were prepared from purified commercial bilirubin-IXα (Compernolle *et al.*, 1971).

As separation of the methylated azo derivatives of mesobilirubin-IXγ could not be achieved, meso derivatives of the methyl esters of azopigments IIIE and IIIF were prepared as follows. Solutions of purified methyl esters of azopigments IIIE and IIIF in pentanone were shaken for 3min at room temperature with H_2 at a pressure of about 81kPa (0.8atm) in the presence of Pd catalyst. After centrifugation the supernatants were decanted and the sediments washed again with pentanone. The combined extracts, containing the meso derivatives of the methyl esters of either azopigment IIIE or IIIF, were concentrated *in vacuo* and chromatographed with benzene/ethyl acetate (17:3, v/v).

Spectrophotometry. Characteristic absorption spectra of purified azopigments in methanol and of bile pigments in dimethylformamide were obtained as described previously (Heirwegh *et al.*, 1975).

Mass spectrometry. The pigments were introduced into the ion source of an AEI MS902S mass spectrometer via the direct-insertion system. The operating conditions of the ion source were: temperature 160–250°C as required, electron voltage 70eV, trap current 500μA, accelerating voltage 8kV.

Tetrakis(trimethylsilyl) derivatives of the four bilirubins were prepared by the method of Salmon & Fenselau (1974). In our slightly modified procedure the bilirubin isomer was eluted from the silica gel with methanol, dried *in vacuo* and dissolved in 30μl of pyridine, and to this solution 10μl of *NO*-bis-(trimethylsilyl)trifluoroacetamide was added and stored in a sealed tube for 1h. The direct-introduction probe was dipped into this solution. Solvent and reagent were evaporated in the direct-introduction lock of the mass spectrometer. No satisfactory mass spectra were obtained for bilirubin-IXβ and -IXδ in this way and the same trimethylsilylation procedure was therefore also applied to the meso derivatives of these tetrapyrroles.

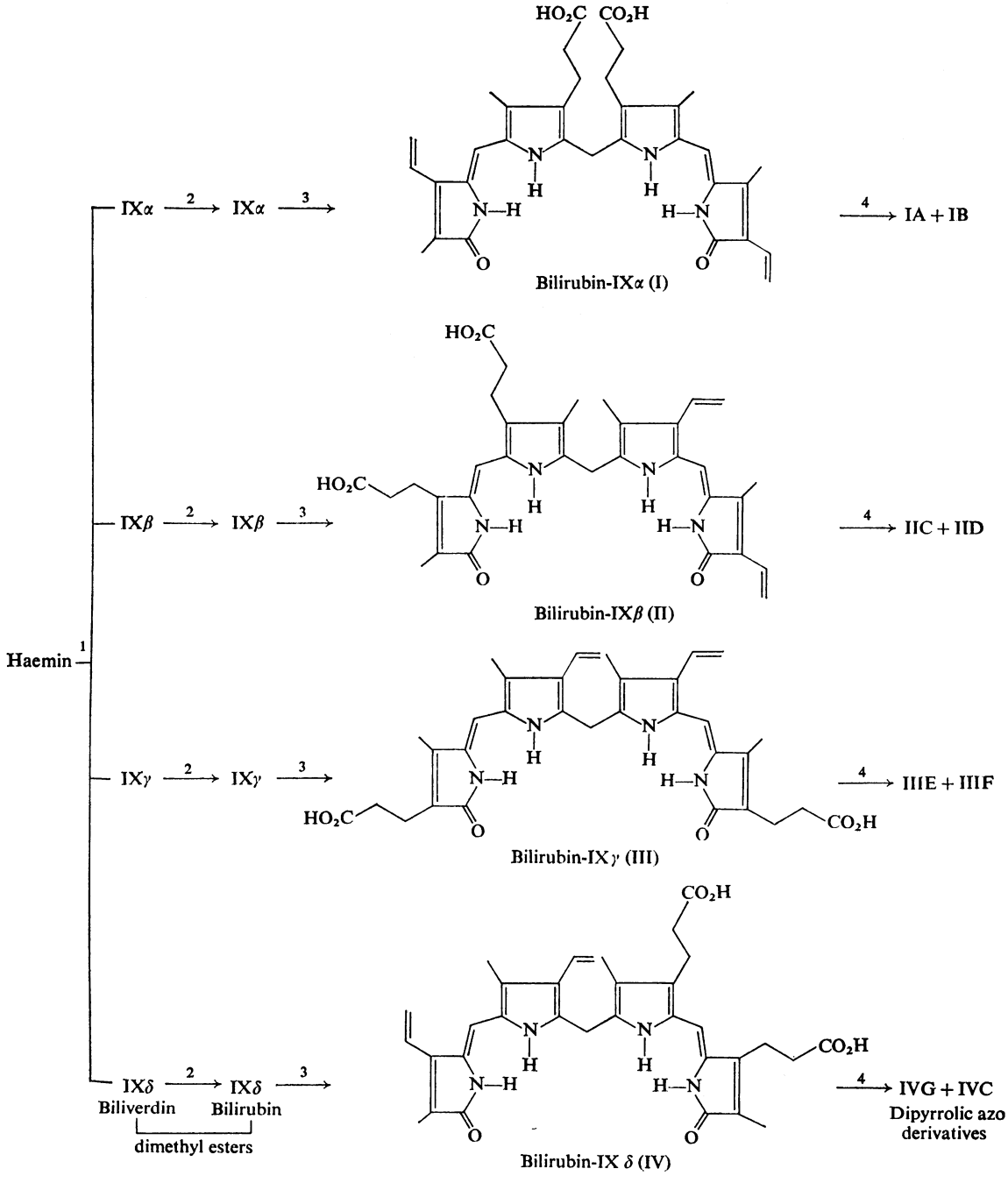
Results and Discussion

Synthesis of the four isomers of bilirubin-IX

The synthesis of the four bilirubin isomers from haemin involves three steps: (1) oxidative cleavage of haemin and separation of the resulting biliverdins as their structurally characterized dimethyl esters (Bonnett & McDonagh, 1973); (2) reduction of the biliverdin esters to the corresponding bilirubin esters; (3) alkaline hydrolysis of the methyl ester groups. The three steps are described in some detail below. Scheme 1 depicts the connexion between individual biliverdin-IX isomers and the corresponding bilirubin-IX isomers.

Preparation and determination of structure of individual isomers of biliverdin dimethyl esters. The formation *in vitro* of a mixture of the four verdins by oxidative cleavage of haemin in aqueous pyridine is well documented (Petryka *et al.*, 1962; O'Carra & Colleran, 1970; Bonnett & McDonagh, 1973). Special attention was paid to the separation and structural characterization of the verdin dimethyl esters, since assignment of the isomeric structures of the rubins synthesized (see below) depends finally on their preparation from known biliverdin-IX isomers. Adequate separation on a preparative scale of the verdin dimethyl esters was obtained (Table 1).

Structure assignment of the separated esters was based primarily on simultaneous analytical t.l.c. with the procedures of O'Carra & Colleran (1970) and of Bonnett & McDonagh (1973). These two systems use different supporting materials and developing solvents and yield completely different migration sequences (Table 1); the respective authors independently validated the structures of the separated


Haemin
1
IXα
2
IXα
3
4
IA + IB
Bilirubin-IXα (I)
IXβ
2
IXβ
3
4
IIC + IID
Bilirubin-IXβ (II)
IXγ
2
IXγ
3
4
IIIE + IIIF
Bilirubin-IXγ (III)
IXδ
Biliverdin
2
IXδ
Bilirubin
3
dimethyl esters
4
IVG + IVC
Dipyrrolic azo derivatives
Bilirubin-IX δ (IV)

	R^1	R^2	R^3	R^4
IA	CH_3	$CH{=}CH_2$	CH_3	$CH_2{-}CH_2{-}CO_2H$
IB	$CH{=}CH_2$	CH_3	CH_3	$CH_2{-}CH_2{-}CO_2H$
IIC (IVC)	CH_3	$CH_2{-}CH_2{-}CO_2H$	$CH_2{-}CH_2{-}CO_2H$	CH_3
IID	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	CH_3
IIIE	$CH_2{-}CH_2{-}CO_2H$	CH_3	CH_3	$CH{=}CH_2$
IIIF	$CH_2{-}CH_2{-}CO_2H$	CH_3	$CH{=}CH_2$	CH_3
IVG	CH_3	$CH{=}CH_2$	CH_3	$CH{=}CH_2$

Scheme 1. *Synthesis of bilirubin-IX isomers and of their ethyl anthranilate azo derivatives*

Step 1: haem is converted into biliverdin-IX isomers by coupled oxidation. After methyl ester formation the four verdin isomers are separated by t.l.c. Step 2: reduction with $NaBH_4$ yields the corresponding rubins. Step 3: they are hydrolysed in alkaline medium to the free acids. Step 4: dipyrrolic azo derivatives are obtained by treatment of the rubins with diazotized ethyl anthranilate. The formulae of the azopigments with the positions of the δ-substituents R^1, R^2, R^3 and R^4 are shown in Scheme 2. IA and IB correspond respectively to the so-called vinyl and isovinyl isomers of azodipyrrole derived from bilirubin-IXα (Jansen & Stoll, 1971).

Table 1. *Properties of the isomers of biliverdin-IX dimethyl esters*

R_F values refer (*a*) to the chromatographic procedure of Bonnett & McDonagh (1973), as slightly modified in the present paper, and (*b*) to the method of O'Carra & Colleran (1970). Absorption spectra were obtained in chloroform solution. R_1 is the ratio E_{380}/E_{650} and R_2 is $E_{max.-3}/E_{max.-4}$. Under the heading M^+ the mass of the molecular ion is indicated with the relative abundance in parentheses. The last column gives in sequence: the ion source temperature, in parentheses, and a series of the more important fragment ions followed, in parentheses, by their relative abundance.

			Absorption spectrum						Mass-spectral data	
	R_F values		Wavelength of maximum (nm)							Important fragment ions
Compound	(*a*)	(*b*)	1	2	3	4	R_1	R_2	M^+	
IXα	0.77	0.11		312	380	643–650	3.22	3.36	610 (100)	(190°C): 595 (5), 579 (3), 551 (3), 537 (7), 523 (7), 463 (5), 450 (4), 326 (10), 314 (6), 313 (10), 311 (9), 305 (M^{2+}, 5), 300 (18)
IXβ	0.82	0.37	280	315	377	650–658	3.62	3.68	610 (100)	(200°C): 595 (6), 579 (5), 551 (6), 537 (9), 523 (7), 521 (4), 388 (7), 360 (65), 266 (50), 251 (50)
IXγ	0.74	0.45	273		376	638	3.11	3.15	610 (100)	(200°C): 595 (12), 579 (8), 537 (8), 326 (20), 314 (8), 313 (6), 312 (8), 311 (11), 300 (100), 285 (20), 269 (15), 241 (25), 240 (45)
IXδ	0.64	0.27	275		376	650–655	2.94	2.98	610 (90)	(195°C): 595 (10), 579 (8), 551 (11), 537 (12), 523 (15), 509 (8), 360 (100)

verdins. Therefore structure assignment to the presently isolated verdins is well supported by their chromatographic behaviour in the two independent t.l.c. systems. The structures were further confirmed by spectrophotometric and mass-spectrometric analysis (Table 1). The mass-spectral data listed for the biliverdin-IX dimethyl esters agree with those reported by Bonnett & McDonagh (1973) for the α, β and γ isomers. The anomalous mass spectrum observed by these authors for biliverdin-IXδ dimethyl ester must have been due to the high ion-source temperature, 260°C. At 195°C we did not observe the reported hydrogenation reaction ($M+2$, *m/e* 612) but found a highly abundant molecular ion (M^+ 610, 90% instead of 6%). The structural similarities between the α and γ biliverdin dimethyl esters are apparent from the common peak at *m/e* 300, whereas the peak at *m/e* 360 is shared by the mass spectra of the β and δ

isomers. The *m/e* 300 and 360 peaks correspond to dipyrrolic fragment ions, substituted with one and two CH_2–CH_2–CO_2CH_3 side chains respectively.

Formation of bilirubin-IX dimethyl esters and saponification. After reduction of individual biliverdin-IX dimethyl esters with $NaBH_4$, the resulting bilirubin esters were submitted to alkaline hydrolysis in methanol/water mixtures. In general, demethylation was complete after 15–20min. For the β isomer the results varied somewhat, but in most experiments hydrolysis was complete within 30min. After acidification to pH2.7 and saturation of the aqueous phase with $(NH_4)_2SO_4$ the bile pigments were extracted into chloroform. When the salting-out reagent was omitted the IXγ isomer remained in the aqueous phase to some extent (Table 2). The colour recovery was even lower when the extraction was done at pH7.4.

Table 2. *Extraction of the bilirubin-IX isomers α and γ*

Methanol solutions of the dimethyl esters of bilirubin-IXα and -IXγ were submitted to alkaline hydrolysis as described in the Materials and Methods section. Six 2ml samples of each solution were mixed with 2ml of chloroform. The mixtures were further treated in duplicate with 4ml of (A) phosphate buffer, pH7.4 (81.8ml of 0.5M-KH_2PO_4 and 59.2ml of 0.5M-NaOH brought to 1 litre), (B) glycine/HCl buffer, pH2.7, or (C) glycine/HCl buffer, pH2.7, saturated with $(NH_4)_2SO_4$. After thorough shaking the mixtures were centrifuged. Absorption in the aqueous and organic phases was measured at 400 and 450nm respectively for bilirubin-IXγ and -IXα. Assuming that the molar extinction coefficients are the same in both phases the amount of bile pigment transferred to the aqueous phase was calculated, taking account of the volume ratio of both phases.

	Percentage of total bile pigment present in the aqueous phase					
	Bilirubin-IXα			Bilirubin-IXγ		
Expt. no.	A	B	C	A	B	C
1	1.4	0.7	0.5	13.0	6.2	2.5
2	1.3	0.7	0.8	15.7	6.4	2.9

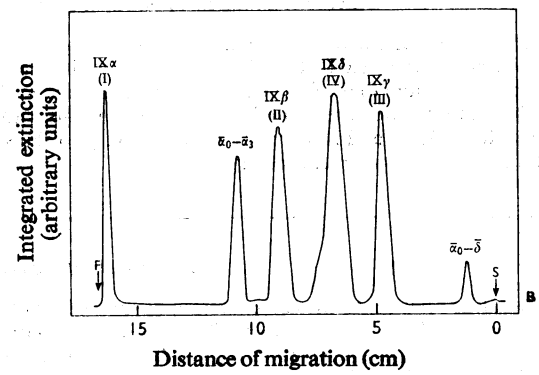


Fig. 1. *Densitogram of a thin-layer chromatogram of the bilirubin-IX isomers*

A mixture of the synthetic bilirubin-IX isomers was developed with chloroform/methanol/water (40:9:1, by vol.). The monoglucoside ($\bar{\alpha}_0$–$\bar{\alpha}_3$) and the monoglucuronide of bilirubin-IXα ($\bar{\alpha}_0$–$\bar{\delta}$), prepared by the method of Heirwegh *et al.* (1975), were included in the mixture to reveal the relative polarities of the pigments. The application line is indicated by S, the solvent front by F, the baseline by B.

Table 3. *Properties of the bilirubin-IX isomers and of their methyl esters*

R_F values are given for the solvent mixtures (*a*) chloroform/methanol/water (40:9:1, by vol.) and (*b*) chloroform/methanol/water (10:5:1, by vol.). Absorption spectra were obtained in dimethylformamide solution. For mass spectrometry the tetrakis(trimethylsilyl) derivatives of bilirubin-IXα and -IXγ and of mesobilirubin-IXβ and -IXδ were used. The masses of the molecular ion (M^+) and of the fragment ions are followed, in parentheses, by their relative abundance.

	R_F value		Wavelength (nm)		Mass-spectral data	
						Important fragment ions
Compound	(*a*)	(*b*)	Maximum	Shoulder	M^+	
Bilirubin-IXα	0.98	0.98	458	430	872 (100)	857 (8), 755 (1), 741 (1), 727 (3), 443 (25), 436 (M^{2+}, 12), 430 (14), 429 (30)
Bilirubin-IXβ	0.55	0.79	400	453		
Mesobilirubin-IXβ					876*	
Bilirubin-IXγ	0.29	0.69	388	427	872 (100)	857 (18), 755 (2), 741 (4), 727 (2), 443 (40), 436 (M^{2+}, 8), 430 (30), 429 (8)
Bilirubin-IXδ	0.41	0.75	398	433		
Mesobilirubin-IXδ					876 (100)	861 (10), 759 (1), 745 (1), 731 (1.6), 561 (15), 548 (9), 547 (8), 438 (M^{2+}, 9), 329 (40), 315 (5)

* The mass spectrum of mesobilirubin-IXβ was not sufficiently abundant to determine relative intensities of fragment ions.

T.l.c. of bilirubin. Does scrambling occur? Mixtures of the four isomers of bilirubin-IX can be separated satisfactorily by t.l.c. (Fig. 1; Table 3). The α isomer moved as purified commercial bilirubin. The non-α isomers are much more hydrophilic and have polarities intermediate between those of bilirubin-IXα monoglucoside and bilirubin-IXα monoglucuronide.

In assigning structures to linear tetrapyrroles containing a central methylene bridge, due attention should be paid to possible scrambling occurring in the course of manipulations (Stoll & Gray, 1970), thereby confusing the issue of structure assignment to the isolated compounds. The risk is particularly high in organic solution containing mineral acid (Bonnett & McDonagh, 1970) and in aqueous solution at neutral to slightly alkaline pH in the presence of O_2 and light (McDonagh & Assisi, 1972*b*). In the present procedures we attempted to keep dipyrrole exchange to a minimum. Thin-layer plates were developed in the dark with neutral solvent systems, in a N_2 atmosphere. Chemical treatments were done in dimmed light on solutions that were continuously flushed with H_2 or N_2. The dimethyl esters of bilirubin-IX isomers were hydrolysed in strongly alkaline medium, as under these conditions dipyrrole exchange does not occur (McDonagh & Assisi, 1972*b*). When acidification was unavoidable slightly acidic buffered solutions were used, the duration of the treatment was kept to a minimum, and the bile pigments were extracted without delay into a suitable organic phase.

Dipyrrole exchange in bilirubin-IXβ and -IXδ is easily monitored, as the resulting tetrapyrroles show widely different R_F values (Fig. 2). This is expected as they contain either four carboxyl groups or none. Isomers IIIα and XIIIα formed from bilirubin-IXα show smaller differences in t.l.c. mobilities, but can still be detected (McDonagh & Assisi, 1971). Tetrapyrroles resulting from dipyrrole exchange were present to insignificant extents in our preparations of IXα, IXβ and IXδ isomers. Thus the isomeric status of these synthetic preparations can be related unequivocally to the structures of the parent biliverdin-IX isomers (Scheme 1). On treatment of compound III with dimethyl sulphoxide/conc. HCl (8:1, v/v), no separation into three bands could be demonstrated. This failure probably reflects a poor separation of the

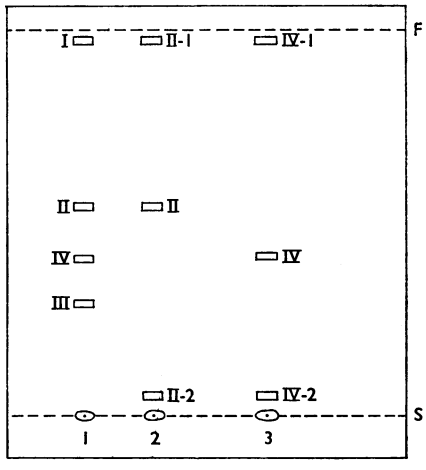


Fig. 2. *Thin-layer chromatogram of tetrapyrroles resulting from scrambling of bilirubin-IXβ and bilirubin-IXδ*

Dipyrrole exchange of preparations of bilirubin-IXβ (track 2) and bilirubin-IXδ (track 3) was induced by treatment with mineral acid by the method of McDonagh & Assisi (1972*a*). The chromatogram was developed with chloroform/methanol/water (40:9:1, by vol.) with reference to a mixture of the synthetic bilirubin-IX isomers (track 1). The Roman numbers I, II, III and IV refer respectively to bilirubin-IXα, -IXβ, -IXγ and -IXδ. The bile pigments present on tracks 2 and 3 were scraped from the plates and the powders were treated with diazotized ethyl anthranilate. The resulting azopigments were analysed by t.l.c. (see the Materials and Methods section). The azo derivatives obtained were: IIC and IID from II, IID from II-1, IIC from II-2, IVC and IVG from IV, IVG from IV-1, and IVC from IV-2. The significance of the symbols used to denote the azopigments is made clear in Scheme 2. The application line is indicated by S, the solvent front by F.

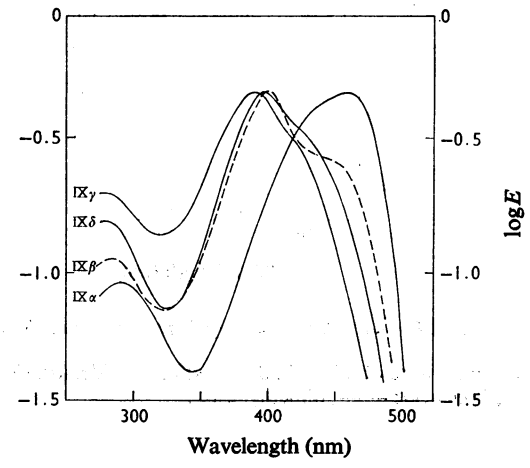


Fig. 3. *Characteristic absorption spectra of the bilirubin-IX isomers in dimethylformamide*

Plotting of the logarithm of the extinction renders the shape of the curve independent of the concentration of the sample and of the optical path-length of the sample cuvette. To facilitate comparison, especially in the visible range, the spectra have been shifted along the vertical axis (log E). The same remarks apply to Figs. 4 and 5.

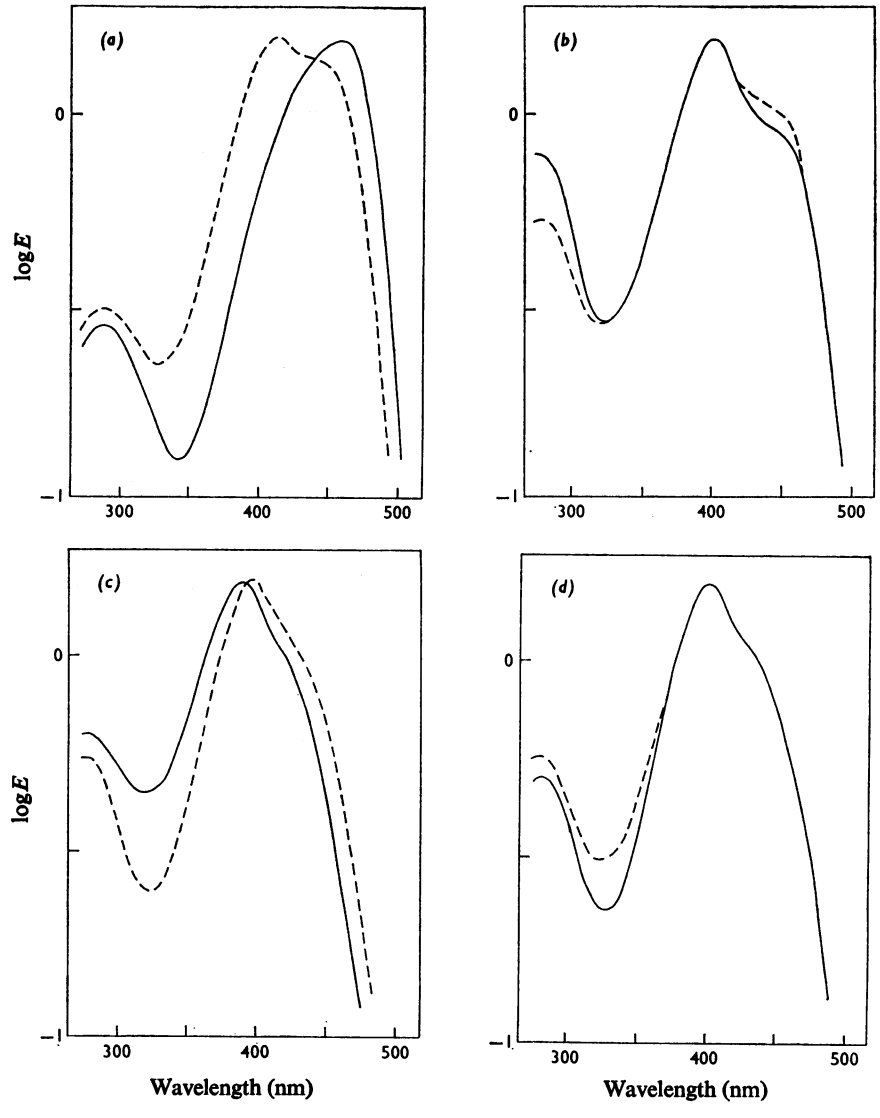


Fig. 4. *Characteristic absorption spectra of the bilirubin-IX isomers and of the corresponding dimethyl esters*

The spectra were obtained in dimethylformamide. (*a*), (*b*), (*c*) and (*d*) are respectively bilirubin-IXα, -IXβ, -IXγ and -IXδ. ——, Free acid; ----, dimethyl ester.

isomers produced. Obviously, although we cannot rigorously exclude the presence of IIIγ and XIIIγ isomers in our preparations of bilirubin-IXγ, important contamination is unlikely, as identical treatments used in the preparation of the IXα, IXβ and IXδ isomers were successful.

Identification of the isomers of bilirubin-IX

Although compounds I, II, III and IV (Fig. 1) could be shown unequivocally to correspond respectively to bilirubin IXα, IXβ, IXγ and IXδ (Scheme 1), independent establishment of the major structural features of the synthetic rubins was sought.

In particular, it is of interest to develop simple diagnostic tests to establish the structures of unknown preparations of bilirubin-IX. Confirmation of the postulated structures (Scheme 1) was obtained both at the tetrapyrrolic stage by mass spectrometry of trimethylsilyl derivatives and spectrophotometry and by formation and analysis of dipyrrolic azopigments.

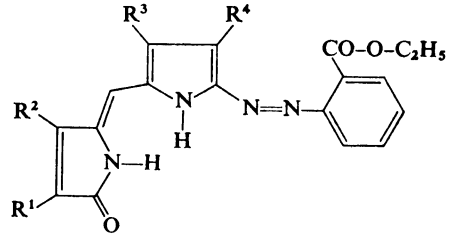
Identification at the tetrapyrrolic stage. Tetrakis-(trimethylsilyl) derivatives of the four bilirubins were prepared by the method of Salmon & Fenselau (1974). The mass spectra of the derivatives of bilirubin-IXα and bilirubin-IXγ are similar, showing some variations in the relative intensities of fragment ions (Table 3). The molecular ions at *m/e* 872 constitute the base peaks in both spectra. Fragmentation about the central methylene bridge gives rise to the ions at *m/e* 429, 430 and 443.

The trimethylsilylation procedure did not yield useful mass spectra for the IXβ and IXδ isomers. The presence of two vinyl groups on one dipyrrole moiety probably induces considerable thermal lability, as indicated by the important increase in thermal stability found for dipyrrolic azopigments when two vinyl groups are converted into ethyl substituents (see below). Accordingly, trimethylsilylation was performed on mesobilirubin IXβ and IXδ, obtained by hydrogenation and t.l.c. separation. The procedure resulted in a characteristic mass spectrum for the IXδ derivative (Table 3). The molecular ion at *m/e* 876 again represents the base peak, and cleavage about the central methylene bridge gives rise to the ions at *m/e* 561, 548 and 547 for the fragments substituted with two CH_2–CH_2–CO_2–$Si(CH_3)_3$ side chains and to the ions at *m/e* 329 and 315 for the fragments with two ethyl groups. A weak molecular ion was observed for the IXβ derivative; apparently very little material survived the consecutive derivative formation and the final evaporation in the mass spectrometer.

Absorption spectra. The spectra of the bilirubin-IX isomers and of their dimethyl esters are characterized by the presence of one maximum and one shoulder between 390 and 460nm (Figs. 3 and 4; Table 3). Typically, bilirubin-IXα showed a maximum at 458nm and a shoulder at about 430nm, whereas for the other isomers the maximum was at 389–400nm with a shoulder at longer wavelengths (Fig. 3). The β isomer is easily differentiated from the γ and δ isomers by a pronounced shoulder at about 453nm. The presence of methylated carboxyl groups has little or no effect on the spectra of the β and δ isomers (Figs. 4*b* and 4*d*), the spectrum of the γ isomer showing a small shift to longer wavelengths (Fig. 4*c*). The spectra of the dimethyl esters of bilirubin-IXγ and -IXδ are very similar from 275 to 350nm and coincided exactly from 350 to 550 nm. Methylation of bilirubin-IXα renders its absorption spectrum qualitatively similar to those of other methyl esters, in particular, to that of the β isomer (Figs. 4*a* and 4*b*).

It is suggested that the particular behaviour of non-esterified bilirubin-IXα is related to its tendency in hydrophobic media to form hydrogen bonds between the carboxyl groups and the –NH.CO– groupings present in non-neighbouring terminal pyrrole rings (Blauer *et al.*, 1972; Kuenzle *et al.*, 1973). By building models of the isomers it was confirmed that multiple hydrogen-bonding is sterically possible for bilirubin-IXα but not for the non-α isomers. The presence of a shoulder at 450nm in the spectra of both methyl esters of bilirubin-IXα and -IXβ could be related to similar systems of five conjugated double bonds starting from the exo vinyl group.

Chromatographic analysis of the azo derivatives and of their methyl esters. In several respects azopigment analysis confirmed the structures postulated for compounds I–IV. Each yielded typical combinations of two azopigments (Schemes 1 and 2). Their chromatographic mobilities in two solvent systems are given in Table 4. In these solvents compounds I and III each yielded a single azopigment spot called respectively Iazo and IIIazo. However, prolonged development with benzene/ethyl acetate (2:3, v/v) on thin-layer plates, provided at the upper edges with a pad of filter paper, resulted in the separation of azopigments Iazo and IIIazo into pairs of closely moving components IA and IB and IIIE and IIIF respectively. Components IA and IB moved respectively as the vinyl and isovinyl isomers of azodipyrrole from commercial bilirubin-IXα. From compounds II and IV mixtures of azopigments IIC and IID and of IVC and IVG were obtained. The derivatives IID and IVG are strikingly less stable and more hydrophobic than the other azopigments. On t.l.c. with benzene/ethyl acetate (17:3, v/v) derivative IID is the more mobile component. In all solvent systems used in the present



Scheme 2. *Dipyrrolic azopigments obtained from synthetic bilirubin-IX isomers*

The Roman numbers I, II, III and IV refer to the parent bilirubins (Scheme 1). IA, IB, IIIE and IIIF are isomers, and so are IID and IVG. IIC is identical with IVC. The nature of the β-substituents R^1, R^2, R^3 and R^4 is defined in Scheme 1. The previously described α_F azopigment corresponds to either IID or IVG or a mixture of the two; azopigment β_x corresponds to IIC (IVC) (Heirwegh *et al.*, 1975).

Table 4. *R_F values of ethyl anthranilate azopigments obtained from bilirubin-IX isomers*

Thin-layer plates were developed with (A) chloroform/methanol (9:1, v/v) (over 1cm) followed by benzene/ethyl acetate (9:1, v/v) (over 18cm), or (B) chloroform/methanol/water (65:25:3, by vol.) (over 1cm) followed by chloroform/methanol (9:1, v/v) (over 18cm).

Parent tetrapyrrole	Azopigment	R_F value A	R_F value B
Bilirubin-IXα (I)	Iazo	0.06	0.64
	IA methyl ester	0.57	
	IB methyl ester	0.65	
Mesobilirubin-IXα	Meso-Iazo	0.06	
	Meso-IA methyl ester	0.52	
	Meso-IB methyl ester	0.58	
Bilirubin-IXβ (II)	IIC*		0.21
	IIC monomethyl ester-1*		0.60
	IIC monomethyl ester-2*		0.51
	IIC dimethyl ester*	0.21	0.87
	IID	0.82	0.93
Mesobilirubin-IXβ	Meso-IID	0.72	
Bilirubin-IXγ (III)	IIIazo		0.51
	IIIE methyl ester	0.42	
	IIIF methyl ester	0.47	
	Meso-IIIE methyl ester	0.44	
	Meso-IIIF methyl ester	0.44	
Bilirubin-IXδ (IV)	IVG*	0.69	0.93
Mesobilirubin-IXδ	Meso-IVG	0.65	

* IVC and its methyl esters had the same R_F values as the corresponding IIC azopigments.

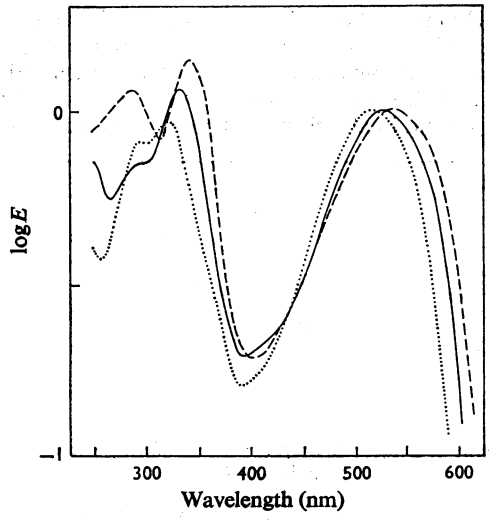


Fig. 5. *Characteristic absorption spectra of dipyrrolic azopigments with various degrees of unsaturation in the β substituents*

No vinyl group, IIC (····); one vinyl group, IB (——); two vinyl groups, IID (– – –). The symbols IB, IIC and IID are explained in Scheme 2.

study derivatives IIC and IVC showed identical chromatographic mobilities.

The presence of carboxyl groups in all azopigments except IID and IVG was indicated by their conversion into more rapidly moving derivatives on treatment with diazomethane (Table 4). Azopigments IA, IB, IIIE and IIIF each yielded a single methyl ester. The methyl esters of IA and IB moved respectively as the methyl esters of the vinyl and isovinyl isomer of azodipyrrole from commercial bilirubin-IXα. From each azopigment IIC and IVC the same major dimethyl ester and the same pair of two minor more hydrophilic side products were obtained. The latter products were transformed into the dimethyl ester by treatment of pentanone solutions with diazomethane. This behaviour is expected for isomeric monomethyl esters of derivatives IIC and IVC.

Optical spectra of the azo derivatives. Three types of azopigment spectra could be recognized, depending on whether the dipyrrole residue contained none, one or two vinyl groups (Fig. 5). Azopigments IID and IVG with two vinyl groups (Scheme 2) typically showed maxima at 535 and 340nm. A third maximum at shorter wavelengths could not be located exactly, owing to changes below 300nm that were probably due to breakdown products of the azopigments. For

Table 5. *Mass-spectral data on ethyl anthranilate azopigment obtained from bilirubin-IX isomers*

The masses of the molecular ion (M^+) and of the fragment ions are followed, in parentheses, by their relative abundances.

Parent tetrapyrrole	Azopigment*	Mass spectral data: M^+	Mass spectral data: Important fragment ions
Bilirubin-IXα (I)	IA	476 (100)	445 (6), 389 (6), 312 (45), 311 (9), 280 (8), 252 (12), 165 (40), 120 (32), 119 (65)
	IB	476 (100)	445 (5), 403 (7), 312 (32), 280 (7), 252 (5), 165 (45), 120 (35), 119 (80)
Mesobilirubin-IXα	Meso IA	478 (100)	447 (5), 405 (15), 314 (85), 282 (30), 165 (30), 121 (15), 120 (13), 119 (40)
	Meso IB	478 (100)	447 (6), 405 (16), 314 (80), 282 (30), 165 (22), 121 (12), 120 (21), 119 (24)
Bilirubin-IXβ (II) and -IXδ (IV)	IIC (IVC)	536 (100)	505 (8), 463 (35), 449 (15), 165 (40), 120 (36), 119 (70)
Bilirubin-IXβ (II)	IID	416	
Mesobilirubin-IXβ	Meso IID	420 (100)	347 (24), 256 (8), 165 (7), 120 (10), 119 (12)
Bilirubin-IXγ (III)	IIIE	476 (5)	403 (1), 312 (0.3), 165 (60), 120 (35), 119 (100)
	IIIF	476 (100)	403 (15), 312 (11), 165 (15), 120 (20), 119 (30)
	Meso IIIE	478 (100)	405 (8), 314 (18), 165 (13), 120 (15), 119 (16)
	Meso IIIF	478 (100)	405 (12), 314 (5), 165 (12), 120 (12), 119 (16)
Bilirubin-IXδ (IV)	IVG	416	
Mesobilirubin-IXδ	Meso IVG	420 (100)	347 (12), 256 (65), 165 (15), 120 (20), 119 (24)

* Except for compounds IID and IVG and their meso derivatives, methyl esters were analysed.

azopigments IIC and IVC and for the meso derivatives listed in Table 4 virtually identical spectra were found between 300 and 600nm with maxima at 294 (0.83), 319 (0.935) and 517nm (1.00), and minima at 301 (0.82) and 392nm (0.145). The extinctions relative to the values at 517nm are given in parentheses. In comparison with the spectra of the two groups of azopigments mentioned above the spectra of the azopigments with one vinyl group were of an intermediate type (Fig. 5). The absorption curve of azopigment IB is shown. Maxima were at 529–530nm (1.00) and 332–333nm (1.2) with a shoulder at shorter wavelengths and a minimum at 393–395nm (0.17). The spectrum of the vinyl isomer IA was shifted slightly to shorter wavelengths. Maxima were at 527 (1.00), 329–330 (1.00) and 295nm (0.66) and minima at 393 (0.17) and 300nm (0.65). Azopigments IIIE and IIIF showed spectra that were similar to those of IB and IA. Esterification of the carboxyl groups had virtually no effect on the spectra.

Mass spectra of the azopigments. The mass spectra (Table 5) of the methyl esters of the isomeric compounds IA, IB, IIIE and IIIF yielded molecular ions at *m/e* 476. Structures have been previously assigned to compounds IA and IB on the basis of their n.m.r. spectra (Compernolle *et al.*, 1971). Structures IIIE (attributed to the less mobile isomer, Table 4) and IIIF were assigned on the basis of the greatly decreased relative intensity of the molecular ion in the spectrum of the methyl ester of compound IIIE. This results from interaction of the exo vinyl with the azo group. Hydrogenation of the vinyl double bond in methyl esters of compounds IIIE and IIIF removed this interaction in the former compound and gave rise to equally abundant molecular ions for the meso derivatives at *m/e* 478. Moreover, characteristic differences in the relative intensities of the $[M-C_6H_4(CO_2Et)NH^{\bullet}]^+$ ions, found at *m/e* 314 in the spectra of the meso esters of compounds IIIE and IIIF, confirm the assigned structures. Indeed, abstraction by an azo nitrogen of a secondary hydrogen from the exo ethyl group in the meso derivative of the methyl ester of compound IIIE is energetically more favourable than the analogous abstraction of a primary hydrogen from the exo methyl group in the meso derivative of the methyl ester of compound IIIF. This results in a greater abundance for *m/e* 314 in the spectrum of the IIIE derivative. The hypothesis of initial hydrogen abstraction from the exo ethyl and exo methyl groups was supported by deuterium-exchange experiments which further established equilibration of the abstracted hydrogen with the hydrogen of the pyrrole nitrogen (F. Compernolle, N. Blanckaert & K. P. M. Heirwegh, unpublished work).

Compounds IID and IVG yielded molecular ions at *m/e* 416, but the pronounced thermolability, induced by the presence of two vinyl substituents, obscured the fragmentation patterns observed. The tetrahydro derivatives of IID and IVG, however,

yielded abundant molecular ions at *m/e* 420 and characteristic mass spectra. Again the relative intensity of the $[M-C_6H_4(CO_2Et)NH^{\bullet}]^+$ ion at *m/e* 256 was appreciably larger for tetrahydro IVG than for tetrahydro IID, confirming the structures postulated on the basis of the respective structures of the precursor bilirubins II and IV.

The azopigments IIC and IVC showed identical spectral properties and t.l.c. mobilities. The mass spectra of the dimethyl (Table 5) and diethyl esters (F. Compernolle, N. Blanckaert & K. P. M. Heirwegh, unpublished work) yielded abundant molecular ions at *m/e* 536 and 564 respectively.

General comments

Determination of the isomeric composition of biliverdin-IX can be done by t.l.c. of methyl ester derivatives (Gray *et al.*, 1972). In the past, analysis of bilirubin-IX isomers has been based on previous conversion into either verdin esters or monopyrrolic oxidation products. The latter procedure does not allow one to distinguish between the IXβ and IXδ isomers. In the former method bilirubin is converted into biliverdin by oxidation in organic medium at acid pH. Under such conditions dipyrrole exchange is likely to occur to some extent, thereby possibly confusing quantification and structure assignment. By using synthetic bilirubin-IX isomers it can be shown that direct separation by t.l.c. is possible (Fig. 1). This has clear-cut advantages for purposes of detection, identification and assay. In general, at most, two steps precede separation: extraction of bile pigments from the biological sample and deconjugation. Hydrolysis in strongly alkaline medium avoids scrambling (McDonagh & Assisi, 1972*b*). On a micro-scale, unequivocal confirmation of the structures of isolated bilirubin-IX isomers can be obtained by various tests. T.l.c. of azo derivatives yields for each bilirubin-IX isomer typical combinations of two azopigments (Schemes 1 and 2; Table 4). In particular, bilirubin-IXβ and -IXδ can be differentiated by the different t.l.c. mobilities of the nonpolar dipyrrolic azo derivatives IID and IVG. Differences in the absorption spectra of the parent rubins can also be exploited (Fig. 3).

In the course of the present work an important shortcoming of the available methodology has become apparent. The bilirubin-IX isomers show pronounced differences in physicochemical properties, such as their chromatographic behaviour (Fig. 1) and distribution in two-phase solvent systems (Table 2). It is likely that, in the past, unawareness of this behaviour has led to partial or even complete loss of some isomers in the course of preparing the samples for analysis. Usually, preparation of unconjugated bilirubin-IX for isomeric analysis has involved extraction from aqueous solutions and subsequent washing of the organic extracts. In contrast with complete extraction of bilirubin-IXα from aqueous solutions at pH7/8 with chloroform (Bratlid & Winsnes, 1971; Brodersen & Vind, 1963) recovery of the other isomers, and in particular of bilirubin-IXγ, was incomplete even at pH3 (Table 2). Extraction is improved by salting-out. Similarly, crystallization of mixed preparations of bilirubin-IX is expected to lead to selective losses of the more hydrophilic non-α isomers. The present observations may prompt a renewed interest in problems related to catabolism of haem such as the study of poorly known pathways that become prominent when the normal metabolism is disturbed.

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