Separation and Structural Analysis of Vinyl- and IsovinyI-Azobilirubin Derivatives

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The coupling reaction of bilirubin with the diazonium salts of ethyl anthranilate or of aniline yields two isomeric azopigments. These can be separated by t.l.c. as their methyl esters. The mass spectra of each pair of azopigments are very similar, showing that they are isomers. Proton-magnetic-resonance spectrometric studies show that they differ in the positions of the substituents on the pyrrolenone end ring; in one compound the methyl and vinyl groups are interposed compared with the other compound. These azo compounds were used as reference standards for determination of the site of conjugation in bilirubin monoglucuronide prepared enzymically. Analysis showed that conjugation occurs at the carboxyethyl side chain of both sides of the bilirubin molecule. During the preparation of the ethyl anthranilate reference compounds a series of minor azopigments were isolated by t.l.c. Analysis of the mass spectra of many of these showed that three side reactions can occur: (1) methylation of the imide carbonyl group; (2) addition of methanol or water to the vinyl substituent; (3) transmethylation of the ethoxycarbonyl group.

The coupling reaction between diazonium salts and bilirubins yields 2 molecules of dipyrrolic azopigments (Fischer & Haberland, 1935; Fischer, Plieninger & Weissbarth, 1941; Overbeek, Vink & Deenstra, 1955). The isomeric reaction products have not, however, been separated and identified. It has since been shown that coupling of bilirubin with the diazonium salt of ethyl anthranilate splits the bilirubin molecule at the central methylene bridge, yielding 2 molecules of ethyl anthranilate azobilirubin (Compernelle, Jansen & Heirwegh, 1970), which could be separated as two closely moving fractions by t.l.c. On mass spectrometry, each gave a molecular ion at m/e 462 and almost identical fragmentation patterns in agreement with the proposed structure of ethyl anthranilate azobilirubin, suggesting that the two fractions were the 'vinyl' and 'isovinyI' isomers. Treatment of each of the free acids with diazomethane yielded methyl esters, which could be separated into variable amounts of two fractions each showing a mass spectrometric molecular ion at m/e 476 and a fragmentation pattern consistent with their being methyl esters of ethyl anthranilate azobilirubin. Compernelle *et al.* (1970) tentatively explained these

findings as the result of lactam–lactim tautomerism, or of syn–anti isomerism caused by the N=N bond or of a change in the conjugated double-bond system. The last two possibilities were suggested by differences in the mass spectra of the two compounds. Kuenzle (1970b) studied the azocoupling reaction of bilirubin with the diazonium salt of aniline. He did not, however, separate the 'vinyl' and 'isovinyI' isomers but characterized spectrometrically the nature of one of them by preparing the phenylazo derivative of vinylneoxanthobilirubin acid.

Separation of the 'vinyl' and 'isovinyI' isomers of azobilirubin could be of importance for the determination of the site of conjugation in bilirubin monoglucuronide and other conjugates. The present paper extends previous work with ethyl anthranilate azopigments and shows that separation of ethyl anthranilate azobilirubin and of its methyl esters into two components is due to vinyl–isovinyI isomerism and not to lactam–lactim, syn–anti or conjugation isomerism. The isomers of aniline azobilirubin methyl ester were also separated and characterized. During the separation procedures trace amounts of numerous other azopigments were found. The nature of many of them has been established by mass spectrometry. Analysis of the

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azobilirubin conjugates derived from rat bile showed that each consists of a mixture of the 'vinyl' and 'isovinyl' isomers.

EXPERIMENTAL

Materials. Bilirubin was from British Drug Houses Ltd. (Poole, Dorset, U.K.) and was used without further purification. Pentan-2-one was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). Ethyl anthranilate was from Kodak Ltd. (London, U.K.). All the following reagents were of analytical grade: chloroform, methanol, ethyl acetate, benzene, acetone, ethanol, sulphuric acid. Aniline (BDH) was used without redistillation. Vinylneoxanthobilirubinic acid methyl ester was a gift from Professor H. Plieninger, Heidelberg, Germany. Bile was obtained from male Sprague-Dawley rats (300–500 g body wt.), fed on a standard diet, after intravenous loading with bilirubin.

T.l.c. plates. Pre-coated silica gel plates (DC-Alufolien 5553/0025) and pre-coated silica gel F254 plates (5729/0050) (E. Merck A.-G., Darmstadt, Germany) were used without any previous treatment. Silica gel G plates were prepared from silica gel G (Merck) by spreading the slurry, obtained by mixing the silica with water, over the plates to a depth of 0.5 mm. The plates were dried in an oven at 130°C. After cooling, no special precautions were taken to keep the plates dry.

Ethyl anthranilate azopigments. These were prepared by coupling bilirubin IX α , dissolved in chloroform, with the diazonium salt of ethyl anthranilate (0.06M in 0.15M-HCl) in the presence of methanol (5:2:10, by vol.), which served as reaction accelerator (Heirwegh, Van Hees, Le Roy, Van Roy & Jansen, 1970). The reaction was allowed to proceed for 45 min. The azopigments were extracted into chloroform by shaking the reaction mixture with water and, after separating the organic phase from the aqueous layer, the organic phase was evaporated to dryness *in vacuo* in a rotary evaporator. The dried pigments were redissolved in chloroform and purified repeatedly on silica gel columns (extra pure, particle size 0.05–0.2 mm; E. Merck A.-G.) as described by Heirwegh *et al.* (1970) until further chromatography caused no changes in the visible- or u.v.-absorption spectrum. Usually three chromatographic cycles were sufficient. The pigments were finally dried and stored in a dark-glass vial.

Methyl esters were prepared by dissolving the pigments in methanol–18M-H₂SO₄ (50:1, v/v). The methylation was allowed to proceed overnight at 45°C or for 4 h at 60°C. Chloroform was then added and the acid removed by the addition of water. The chloroform extract was washed three times with water and the pigments were evaporated to dryness *in vacuo*.

Abbreviations. The notation α , α_0 , β , γ and δ used throughout the text is identical with that introduced by Heirwegh *et al.* (1970). The Greek letter α is used for ethyl anthranilate azobilirubin prepared from bilirubin; α_0 , β , γ and δ refer to the different azopigment groups isolated chromatographically from ethyl anthranilate azopigments derived from bile. α_0 is identical with α ; δ has been shown to be the β -D-monoglucuronide of ethyl anthranilate azobilirubin. β and γ are uronic acid-containing acyl glycosides of ethyl anthranilate azobilirubin. Methyl esters of these pigments are indicated by the subscript

m, e.g. α_m refers to the methyl ester of ethyl anthranilate azobilirubin.

T.l.c. of pigments. (a) Ethyl anthranilate azopigments (α). These were subjected to multiple development chromatography on silica t.l.c. plates (either DC-Alufolien or F254 plates) in chloroform–ethyl acetate (1:1, v/v) as described by Compennolle *et al.* (1970). After six developments, with intermediate drying of the plates, the ethyl anthranilate azobilirubin pigment separated into two closely moving bands.

(b) Methyl esters of ethyl anthranilate azobilirubin. These were chromatographed on a preparative scale (5–10 mg on each plate) on 0.5 mm-thick silica gel G plates (Merck) with chloroform–benzene (1:1, v/v). The plates were developed over 20 cm, dried and rechromatographed in the same solvent system. After three developments, the main pigment band separated into two fractions of approximately equal intensities, which were separated by a white zone 0.5–1 cm wide. Numerous minor bands were also present, which were more polar than the main azopigment fractions. The different pigment bands were scraped off, eluted from the silica with chloroform and evaporated to dryness under N₂. Homogeneity of the main pigment fractions was checked by rechromatography of small amounts of each fraction on similar plates by using the same solvent system and also 1,2-dichloroethane.

Preparation of ethyl anthranilate azobilirubin methyl esters derived from bilirubin XIII α and vinylneoxanthobilirubinic acid methyl esters. The methyl esters of bilirubin IX α and XIII α were prepared by the method of Bonnet & McDonagh (1970) by refluxing commercial bilirubin in methanol–acidic ferric chloride, which, as well as oxidation, causes fission at the central methylene bridge, followed by recombination of the dipyrrolic fragments yielding verdins. The verdins were separated by t.l.c. and then reduced with borohydride to yield the corresponding bilirubins as their methyl esters (M. S. Stoll, unpublished work). The purity of the symmetrical bilirubin ester was checked by mass spectrometry and by comparison, by t.l.c., of its verdin oxidation product with biliverdin XIII α dimethyl ester. Its exact nature was established by using proton-magnetic-resonance spectrometry. Insufficient bilirubin III α dimethyl ester for study was formed by the reaction. Azopigment formation of bilirubin XIII α dimethyl ester was performed as described for bilirubin IX α . Before t.l.c., the resulting ethyl anthranilate azopigments were adsorbed on a column of silica gel (extra pure, bead size 0.5–0.02 mm; Merck) from benzene. After washing with 20 ml of benzene, the azopigments were eluted by using increasing concentrations of chloroform in benzene. The 'vinyl' isomer of ethyl anthranilate azobilirubin methyl ester was synthesized by diazocoupling of vinylneoxanthobilirubinic acid methyl ester. The resulting azopigment was purified as indicated for the ethyl anthranilate azopigments derived from the symmetrical bilirubin XIII α dimethyl ester.

Azopigments from rat bile. These were prepared as described by Heirwegh *et al.* (1970). T.l.c. of the pentan-2-one azopigment extract was performed on silica gel plates (DC-Alufolien). An initial development over 7 cm with chloroform was followed, after drying, by development over 20 cm with chloroform–methanol–formic acid (85:15:1, by vol.). Each azobilirubin fraction belonging

to the α , β , γ and δ groups was converted into the methyl ester of azobilirubin by using methanol-18M- H_2SO_4 (50:1, v/v) and was chromatographed on silica plates in chloroform-benzene (1:1, v/v).

Bilirubin monoglucuronide. This was prepared enzymically by incubating a digitonin-activated homogenate of rat liver with bilirubin and UDP-glucuronic acid (Black, Billing & Heirwegh, 1970). The ethyl anthranilate azopigments were formed and separated by t.l.c. The α and δ pigments (Heirwegh *et al.* 1970) were converted into the methyl esters of ethyl anthranilate azobilirubin and chromatographed in the chloroform-benzene system.

Aniline azopigments. These were prepared from bilirubin and from bile in essentially the same way as the ethyl anthranilate azopigments. The diazonium salt of aniline was prepared as described by Kuenzle (1970a). All pigment fractions were chromatographed in the same systems as the ethyl anthranilate azopigments. Separation of the isomeric methyl esters of aniline azobilirubin was achieved by chromatographing them on silica gel G (Merck) plates, by using chloroform as the solvent system.

Acid hydrolysis of ethyl anthranilate azobilirubin methyl esters. To a small amount of each ethyl anthranilate azobilirubin isomer methyl ester 10 ml of 10M-HCl was added. The 'vinyl' isomer seemed to dissolve readily and gave an intense dark-blue colour. The 'isovinyl' isomer gave a weakly coloured blue solution, most of the pigment remaining undissolved. Each pigment fraction was incubated at 42°C for 16 h and extracted afterwards by addition of chloroform and water. After several washings, the chloroform extract was concentrated *in vacuo* and the pigments belonging to each isomer were chromatographed in chloroform-ethyl acetate (1:1, v/v) by using authentic ethyl anthranilate azobilirubin as reference material.

Spectrometry. (a) Visible spectra. Visible spectra were recorded at room temperature on a Unicam SP.500 spectrophotometer, in chloroform or methanol or pentan-2-one, by measuring extinction at 10 nm intervals and at 2-5 nm intervals in the regions of maximum absorption.

(b) Mass spectra. Mass spectra were recorded by Mr D. M. Carter of the University of London Mass Spectrometry Service, at the School of Pharmacy, on an A.E.I. MS902 double-focusing mass spectrometer (A.E.I., Manchester, U.K.). The samples were inserted on a direct probe. When only very small amounts (<0.1 mg) of the pigments were available, they were dissolved in a drop of chloroform and the 'syrup' obtained was scraped into the probe, and evaporated in the mass spectrometer. The temperature of the ion source was 200°C for all samples, except when otherwise stated. The spectra were run at an ionizing voltage of 70 eV, 6-8 kV accelerating voltage. Normalized histograms were prepared from each mass spectrum.

(c) Proton-magnetic-resonance spectrometry. The proton-magnetic-resonance spectra were recorded by Mr P. N. Jenkins of Imperial College, London, with a Varian HA-100 100 MHz spectrometer. For the spectra of the ethyl anthranilate fractions obtained after separations in chloroform-ethyl acetate (1:1, v/v) a computer of average transients was used and spectra were obtained from the accumulation of 10 separate scans in each case. Spectra were run in deuterated chloroform

at 35°C with tetramethylsilane as internal standard. Only frequency sweep was used. Chemical shifts are given in p.p.m., the τ scale being used. Determination of the exact τ value was obtained from the combined data of the internal reference signals of tetramethylsilane and of chloroform, the latter being at 2.8 τ . Scale expansion was done for each particular segment of the spectra so as to give valuable resolution of some signals.

(d) I.r. spectrometry. I.r. spectra were recorded on a Unicam SP.200 infrared spectrometer. About 0.5-1.0 mg of azopigment was used to prepare the KBr discs. The i.r. spectra of the methyl esters of the aniline azopigments were also recorded in chloroform solutions at different concentrations by using NaCl optics. Determination of the exact ν value was obtained by using the characteristic frequencies of polystyrene.

RESULTS AND DISCUSSION

Methyl esters of ethyl anthranilate and of aniline azopigments

Methylation in methanol-sulphuric acid (50:1, v/v) converted ethyl anthranilate azobilirubin into less-polar derivatives. T.l.c. of the methylated reaction products in chloroform-benzene (1:1, v/v) yielded two major fractions in approximately equal amounts, which together accounted for 93% of the total pigments present. The remaining 7% consisted of a series of pigments that were more polar than the two major fractions. A similar separation of the aniline azopigment methyl esters was obtained when chromatographed with chloroform as the solvent system.

Mass spectrometry. The mass spectra of the two major ethyl anthranilate and aniline azopigment methyl esters (called in order of decreasing R_F value $\text{EA}\alpha_{\text{M1}}$, $\text{EA}\alpha_{\text{M2}}$ and $\text{ANIL}\alpha_{\text{M1}}$, $\text{ANIL}\alpha_{\text{M2}}$ for the ethyl anthranilate and aniline azopigment methyl esters respectively) showed molecular ions at m/e 476 for the ethyl anthranilate azopigments and at m/e 404 for the aniline azopigments (Fig. 1). The fragmentation patterns were consistent with the theoretical structures of ethyl anthranilate and aniline azobilirubin methyl esters (compounds I and II; III and IV, Scheme 1). Although the four spectra were recorded under identical conditions slight differences could be observed. The spectra of $\text{EA}\alpha_{\text{M1}}$ and of $\text{ANIL}\alpha_{\text{M1}}$ showed molecular ions at m/e 476 and 404 respectively (relative ion intensity approx. 50%). The base peak was recorded for ions at 165 and 93 respectively, resulting from cleavage of the N=N bond with migration of 2 hydrogen atoms to the anthranilic ester or aniline part of the molecule respectively; the dipyrrolic fragment ion appears at m/e 312 in all spectra. The molecular ions at m/e 476 and 404 were found to be the base peaks for $\text{EA}\alpha_{\text{M2}}$ and $\text{ANIL}\alpha_{\text{M2}}$. Fragmentation of the methoxycarbonyl ethyl side chain of the ethyl anthranilate azopigments results

in the formation of ions at m/e 445 and 403 by loss of methoxyl and methoxycarbonylmethyl groups respectively. A similar fragmentation pattern was observed for the aniline azopigments giving ions at m/e 373 and 331. The fragment ion at m/e 312 resulting from cleavage of the azopigments at the N=N bond undergoes additional fragmentation with loss of methanol, and of methoxycarbonyl and methoxycarbonylmethyl groups plus 1 hydrogen atom each to give ions at m/e 280, 252 and 238 respectively. The ion at m/e 165 in the ethyl anthranilate azopigment spectra loses either an ethoxyl group or ethanol to give abundant ions at m/e 120 and 119 respectively. Ions at m/e 389 and 317 for ethyl anthranilate and aniline compounds respectively are consistent with the complete loss of

the methoxycarbonylethyl side chain and were seen only in the mass spectra of the more-polar (EA α_{M2}) fractions. Accurate mass measurements of these ions gave 389.1584 (Calc. for $C_{22}H_{21}O_3N_4$ 389.1612) and 317.1385 (Calc. for $C_{19}H_{17}O_1N_4$ 317.1400) respectively. This phenomenon, first observed for the ethyl anthranilate azobilirubin methyl esters, has been discussed by Compennolle *et al.* (1970), who suggested it was the result of either syn-anti isomerism caused by the N=N bond or of vinyl-isovinyl isomerism or of a change in the conjugated double-bond system. The last possibility can be ruled out on the basis that a shift in the conjugated double-bond system would result in a shift either in the visible- or u.v.-absorption maxima, which were similar for both ethyl anthranilate and both

Fig. 1. Normalized histograms of the mass spectra of azobilirubin methyl esters. (a) $\text{EA}\alpha_{\text{M1}}$; (b) $\text{EA}\alpha_{\text{M2}}$; (c) $\text{ANIL}\alpha_{\text{M1}}$; (d) $\text{ANIL}\alpha_{\text{M2}}$.

aniline isomers when the absorption spectra of each pair of isomers were recorded in the same solvent (Table 1). Slight differences undoubtedly reflect the different conjugation of the vinyl group in the two isomers. In the 'isovinyl' isomers this group is linearly conjugated with the chromophore but in the 'vinyl' isomers it is cross-conjugated. Accordingly, where there is a difference in the spectra, the absorption maxima of the 'isovinyl' isomers is shifted bathochromically compared with the 'vinyl' isomers. The structure of the isomeric components could not be determined by mass spectrometry alone.

Proton-magnetic-resonance spectrometry. The proton-magnetic-resonance spectra of the two ethyl anthranilate methyl esters and of the two aniline azobilirubin methyl esters are shown in Fig. 2. The signals in the range 7.9–8.0 τ are within the region characteristic for methyl groups attached to double-bond systems (Jackman & Sternhell, 1969) and these therefore represent β -methyl substituents. In the spectrum obtained from $\text{EA}\alpha_{\text{M1}}$ two signals at 7.91 and 8.01 τ respectively are each due to three protons. $\text{EA}\alpha_{\text{M2}}$ yields signals at 7.99 and 8.01 τ , having a combined integral due to six protons. From the less- ($\text{EA}\alpha_{\text{M1}}$) to the more-polar ($\text{EA}\alpha_{\text{M2}}$)

Scheme 1. Compound I: 2'-(2,5-dihydro-3-methyl-5-oxo-4-vinyl-1H-pyrrol-2-ylidenemethyl)-5'-(2-ethoxy-carbonylphenylazo)-4'-(2-methoxycarbonyl-3'-methylpyrrole. Compound II: 2'-(2,5-dihydro-4-methyl-5-oxo-3-vinyl-1H-pyrrol-2-ylidenemethyl)-5'-(2-ethoxycarbonylphenylazo)-4'-(2-methoxycarbonyl-3'-methylpyrrole. Compound III: 2'-(2,5-dihydro-3-methyl-5-oxo-4-vinyl-1H-pyrrol-2-ylidenemethyl)-5'-(phenylazo)-4'-(2-methoxycarbonyl-3'-methylpyrrole. Compound IV: 2'-(2,5-dihydro-4-methyl-5-oxo-3-vinyl-1H-pyrrol-2-ylidenemethyl)-5'-(phenylazo)-4'-(2-methoxycarbonyl-3'-methylpyrrole. Broken lines indicate a step not performed in practice.

Table 1. *Absorption maxima of the 'vinyl' and 'isovinyl' isomers of ethyl anthranilate and aniline azobilirubin methyl esters*

Solvent	$\lambda_{\max.}(\text{nm})$		
	Pentan-2-one	Chloroform	Methanol
Aniline 'isovinyl' isomer	522	530 336	520 330
Aniline 'vinyl' isomer	520	530 336	518 330
Ethyl anthranilate 'isovinyl' isomer	530	530 335	530 335
Ethyl anthranilate 'vinyl' isomer	528	530 335	530 330

compound (compounds I and II) there is thus an upfield shift of one of the methyl signals, the signal at 8.01 τ remaining constant. The upfield shift of the methyl signal at 7.99 τ present in the spectrum of $\text{EA}\alpha_{\text{M}2}$ indicates a higher shielding of the protons of one of the β -methyl substituents, which is therefore located outside the molecule (Stoll & Gray, 1970). A corresponding downfield shift can be observed in the β -vinyl multiplet signals; those in the region 3.35–4.61 τ ($\text{EA}\alpha_{\text{M}1}$) are upfield compared with those at 3.30–4.49 τ in the spectrum of the other isomer ($\text{EA}\alpha_{\text{M}2}$). This indicates in the latter isomer a lower shielding and represents a vinyl group attached to the inner β -position of the pyrrolenone end ring. This allows assignment of the structure of vinyl ethyl anthranilate azobilirubin to the isomer $\text{EA}\alpha_{\text{M}2}$ (compound II) and of isovinyl ethyl anthranilate azobilirubin to the isomer $\text{EA}\alpha_{\text{M}1}$ (compound I).

The signals due to the vinyl groups in the two isomers have been analysed by spin decoupling. The results further support the assigned positions of the vinyl groups. In the spectrum of the 'vinyl' isomer the signals of the geminal β -protons appear as two overlapping doublets, centred at about 4.37 and 4.39 τ respectively, with a small additional splitting caused by a small geminal coupling constant estimated to be about 1.5 Hz. The α -proton signal appears as a quartet, centred at about 3.44 τ , collapsing to a singlet during irradiation at 4.39 τ , due to removal of the *cis* and *trans* couplings, which were about 12 and 16 Hz respectively. In the spectrum of the 'isovinyl' isomer the signal of the *trans* geminal β -proton has shifted downfield by about 1 τ relative to the *cis* geminal β - and the α -protons, so that it now appears close to the signal of the α -proton. The signal of the *cis* geminal β -proton appears as a quartet centred at about 4.55 τ , that of the *trans* geminal β -proton as a quartet centred at about 3.75 τ and that of the α -proton as a quartet centred at about 3.48 τ . Irradiation at about 4.5 τ caused the low-field quartets to collapse to doublets by removal of the geminal and the *cis* couplings, which were about

3 Hz and 10 Hz respectively. The *trans* coupling was about 17.5 Hz. The large downfield shift of the *trans* geminal β -proton signal is explained by deshielding of this proton by the neighbouring end-ring carbonyl group, only possible if the vinyl group is in the outer position of this ring.

In the spectra of both isomers the α - and β -methylene protons of the methoxycarbonylethyl side chain give rise to a multiplet of an A_2B_2 system centred at about 7.27 τ . A very sharp singlet at 6.42 τ due to three protons is given by the methoxyl protons of the methyl ester group. A relatively intense signal due to one proton at 2.82 τ is due to a trace of chloroform. In the vinyl region, a singlet at 4.21 and at 4.12 τ for compounds I and II respectively representing one proton is due to the olefinic proton of the methene group linking the pyrrolenone and pyrrole rings. At very low field two broad signals at -0.40 and -3.45 τ and at -0.40 and -3.29 τ for compounds I and II respectively could not be interpreted with certainty. Presumably the signals at about -0.40 τ are due to the pyrrole ring NH proton. The signals at -3.45 and -3.29 τ for compounds I and II respectively are unexpectedly found at even lower field and could therefore be due to a lactim OH proton. An alternative explanation for their downfield position is the involvement of the pyrrolenone end ring NH proton in hydrogen bonding as in enols (Jackman & Sternhell, 1969). This downfield shift was not observed in the proton-magnetic-resonance spectrum of synthetic vinylneoxanthobilirubin acid methyl ester. The two ring NH protons gave signals at -0.41 and at -1.41 τ . In the aniline spectra the pyrrole and pyrrolone end ring NH proton signals were not observed. Further analysis of the ethyl anthranilate spectra shows a triplet ($J = 7$ Hz) with centre at 8.60 τ , corresponding to the methyl protons of the ethyl anthranilate ethyl group. The corresponding methylene quartet ($J = 7$ Hz) was found centred at 5.5 τ . The phenyl protons (range 2.02–3.20 τ) of the ethyl anthranilate residue are slightly shifted downfield in agreement with an *o*-substituted phenyl ring.

Fig. 2. Proton-magnetic-resonance spectra of azobilirubin methyl esters, in deuteriochloroform, at 100MHz. TMS, tetramethylsilane. (a) $EA\alpha_{M1}$; (b) $EA\alpha_{M2}$; (c) $ANIL\alpha_{M1}$; (d) $ANIL\alpha_{M2}$; (e) and (f) show spin decoupling of vinyl groups of $EA\alpha_{M1}$ and $EA\alpha_{M2}$ respectively.

The proton-magnetic-resonance spectra of the aniline azopigments were similar to those of the ethyl anthranilate azopigments and showed similar differences in the chemical shifts of the signals from the β -methyl and β -vinyl substituents. The methyl signals were at 7.99 and 8.01 τ in the 'isovinyl' isomer and at 8.04 and 8.26 τ in the 'vinyl' isomer (ANIL α_{M1} and ANIL α_{M2} respectively). Comparison of the two aniline azobilirubin spectra clearly indicates that the separation of the methyl esters of aniline azobilirubin is due to vinyl-isovinyl isomerism.

Evidence against a lactim structure for azobilirubin is given by the i.r. spectra. A possible lactam carbonyl stretching frequency at 1685 cm^{-1} is found in the i.r. spectra of both aniline azobilirubin methyl esters when recorded in potassium bromide discs. This signal does not disappear when the spectra are recorded at different concentrations in chloroform solutions by using sodium chloride optics. Kuenzle (1970b) assigned the carbonyl stretching frequency of a pyrrolenone as a signal at 1650 cm^{-1} . The spectrum of his compound was, however, recorded in Nujol mulls, which might explain the frequency shift. Further evidence for the lactam structure of azobilirubin is given by the difficulty of forming the methyl ether of azobilirubin methyl ester upon treatment with diazomethane. This difficulty is also encountered with the formation of the dimethyl ether of bilirubin dimethyl ester by using the same methylation procedure (Fischer *et al.* 1941). The i.r. spectra of the ethyl anthranilate azopigments could not be interpreted because of the presence of the additional carbonyl group.

Azobilirubin methyl esters from the synthetic compounds

T.l.c. of the azobilirubin methyl esters derived from the methyl ester of vinylneoxanthobilirubinic acid and bilirubin XIII α dimethyl ester showed that they were homogeneous and indistinguishable from one another and from EA α_{M2} . Their identity was further proved by the proton-magnetic-resonance spectra, which were the same as that of EA α_{M2} .

Acid hydrolysis of ethyl anthranilate azobilirubin methyl esters

'Isovinyl' isomer. Only one-third of the 'isovinyl' isomer was hydrolysed by 10M-hydrochloric acid. The remainder was shown to be unaltered methyl ester when chromatographed with the untreated corresponding 'isovinyl' isomer. The hydrolysed pigment had the same R_F value as the more-polar ethyl anthranilate azobilirubin fraction upon t.l.c. with chloroform-ethyl acetate (1:1, v/v).

Attempts to increase yield during acid hydrolysis of the isovinyl ethyl anthranilate azobilirubin methyl ester by heating the reaction mixture failed and caused destruction of the compound, presumably by breaking of the N=N bond, as yellow pigments appeared in the solution. Acid hydrolysis with concentrated sulphuric acid produced a very polar product which could not be re-esterified by using either diazomethane or methanol-18M-sulphuric acid (50:1, v/v).

'Vinyl' isomer. The 'vinyl' isomer methyl ester was completely hydrolysed and gave rise to three products on t.l.c. with chloroform-ethyl acetate. The major product had the R_F value of the less-polar ethyl anthranilate isomer. Mass spectrometry of this pigment showed it to be identical with ethyl anthranilate azobilirubin. In addition two new pigments were found, which were more polar than ethyl anthranilate azobilirubin. One had a pink colour and showed a molecular ion at m/e 480. Its fragmentation pattern showed that it was due to the addition of 1 molecule of water to the vinyl group. The blue component could not be identified as its molecular ion was missing and the spectrum was very weak. After re-esterification of the major product resulting from the acid hydrolysis of the 'vinyl' isomer a single product, with the R_F value of vinyl ethyl anthranilate azobilirubin methyl ester, was found. A corresponding result was found for the hydrolysed fraction of the 'isovinyl' isomer.

Separation of ethyl anthranilate azobilirubin into components

Ethyl anthranilate azobilirubin chromatographed on thin-layer plates can be separated into two closely moving fractions, which on mass-spectrometric analysis show molecular ions at m/e 462 and almost identical fragmentation patterns (Compennolle *et al.* 1970). Preparative chromatography of azobilirubin provided sufficient material for the study of these components. The two fractions were converted into their methyl esters by treatment with methanol-sulphuric acid (50:1, v/v) and their proton-magnetic-resonance spectra were recorded by using a computer of average transients. The spectrum of the methyl ester of the more-polar free azobilirubin component was identical with that of isovinyl ethyl anthranilate azobilirubin methyl ester. The proton-magnetic-resonance spectrum of the methyl ester of the less-polar free azobilirubin compound gave an intense signal at 7.87 τ and a small signal at 7.78 τ , indicating the presence of both positional isomers. This was confirmed by t.l.c. of the methyl ester used for proton-magnetic-resonance spectrometry, which showed the presence of about 20% of the 'isovinyl'

isomer. Thus chromatography of mixtures of the free azobilirubin isomers results in incomplete separation; the less-polar component (predominantly the 'vinyl' isomer) contained about 20% of the 'isovinyl' isomer, whereas the more-polar component (predominantly the 'isovinyl' isomer) contained only traces of the 'vinyl' isomer.

This study shows that the chromatographic separation into two components of both ethyl anthranilate azobilirubin and of its methyl ester is due to vinyl-isovinyl isomerism. The chromatographic order of the vinyl-isovinyl isomers is reversed when the chromatographic behaviour of the methyl esters is compared with that of the free acids. Study of the proton-magnetic-resonance spectra of each fraction clearly showed only two isomers differing in the positions of the β -methyl and β -vinyl groups. This does not exclude the possibility, however, that in addition to vinyl-isovinyl isomerism, syn-anti isomerism induced by the N=N bond (Hantzsch, 1894; Zollinger, 1961) can occur.

Multiple chromatographic development (seven times) of the 'isovinyl' isomer of ethyl anthranilate azobilirubin methyl ester resulted in partial separation into two components. The proton-magnetic-resonance spectra of these components showed no significant differences from one another or from the spectrum of their mixture. It is suggested that these components are syn-anti isomers. If this is so, failure to separate the 'vinyl' isomer into components in a similar manner was probably due to lack of resolving power in the chromatographic system.

Minor components formed during the preparation of azobilirubin methyl esters

Chromatography of large amounts of ethyl anthranilate azobilirubin in chloroform-ethyl acetate (1:1, v/v) showed that in addition to the major components, trace amounts of other pigments were present. The phenomenon was more pronounced when the methyl esters were chromatographed and some of them might be the result of the acid-catalysed methylation process. These pigments was scraped from the plates, eluted and concentrated. Preparative rechromatography of this mixture of pigments yielded 10 groups of pigments, the colours of which varied from red to blue. Some yellow pigments were also seen. Each of the bands was scraped off, the pigments were eluted from the silica with chloroform-methanol (19:1, v/v), evaporated to dryness and, after spotting on t.l.c. plates, rechromatographed repeatedly in chloroform-benzene (1:1, v/v). Finally about 35 bands were seen, some of them present in minute amounts. The fractions with corresponding R_f value and with

similar colour, seen on different plates, were scraped off, eluted, combined and evaporated to dryness. Mass spectra were obtained from several components (Fig. 3). Analysis showed that three main reactions occurred: (1) addition of methanol or water to the vinyl substituent, (2) transmethylation of the ethoxycarbonyl group and (3) methylation of the imide carbonyl group (Fig. 4). One or more of these reactions occurred separately or in combination. In addition the two positional isomers of these products were present, explaining therefore the rather large number of minor compounds. The spectrum of some compounds could not be interpreted as the product from which it was recorded did not appear to be homogeneous.

In all the minor components the most frequently observed change was the addition of methanol to the vinyl group, as is to be expected from Markownikow's rule. This compound with molecular ion at m/e 508 showed a loss of methanol or of a methoxyl group to give ions at m/e 476 and 477 respectively. The ion at m/e 344 was formed by fission of the molecular ion at the N=N bond and further fragmented with loss of methanol or a methoxyl group to give ions at m/e 312 and 313 respectively. No loss of water was observed, indicating that a methoxyl group and a proton are added to the vinyl group (Fig. 4). This was confirmed by acetylation experiments with pyridine-acetic anhydride; the mass spectrum of the resulting pigment was found to be unchanged.

Transmethylation of the ethoxycarbonyl group of the anthranilic ester part of the molecule (reaction 3) together with hydration of the vinyl group (reaction 2) gave a compound with a molecular ion at m/e 480, readily losing water in the mass spectrometer to yield a base peak at m/e 462. That the structure corresponding to the latter ion contains a methoxycarbonyl ethyl and not a carboxyethyl group is demonstrated by the presence of the ion at m/e 312, present only in the spectra of azobilirubin methyl esters; the free acids have a corresponding ion at m/e 298. The prominent ion at m/e 151 represents the methyl anthranilate fragment ion.

The products showing methylation of the imide carbonyl group also had addition of methanol to the vinyl groups and therefore the molecular ion was found at m/e 522. The ions at m/e 491 represented loss of a methoxyl group and the prominent ion at m/e 460 represented loss of two methoxyl groups from the molecular ion. The latter fragmentation could occur only if the imide carbonyl group was methylated. Ions at m/e 358 and at 296 represent loss of the anthranilic ester group from the molecular ion and the ion at m/e 460 respectively. The colour of these compounds was purple, compared with the red colour of the ethyl anthranilate azobilirubin.

Fig. 3. Normalized histograms of the mass spectra of three minor components after (a) addition of methanol, (b) transmethylation and (c) methylation of the imide carbonyl group.

Fig. 4. Type and sites of side reactions occurring in azobilirubin methyl esters. (1) Addition of methanol or water to the vinyl group. (2) Transmethylation of the ethoxycarbonyl group. (3) Methylation of the imide carbonyl group.

Vinyl-isovinyl isomeric composition of azobilirubin conjugates

Ethyl anthranilate and aniline azopigments derived from rat bile showed on t.l.c. (DC-Alufolien silica gel) a similar pattern. No qualitative differences could be observed between the two types of azopigments when the plates were developed in chloroform-methanol-formic acid (85:15:1, by vol.) Diazo coupling of bilirubin conjugates with the diazonium salt of aniline appeared less complete, although azobilirubin conjugates belonging to the α -, β -, γ - and δ -groups were present. For the rat bile studied, α_0 and δ pigments were the major fractions. Separate treatment of each pigment group with methanol-18M-sulphuric acid (50:1, v/v) converted all the fractions into the methyl esters of either aniline or ethyl anthranilate azobilirubin. T.l.c. of these esters invariably yielded two spots with very similar R_F values. Co-chromatography with appropriate reference compounds showed that the separation was due to vinyl-isovinyl isomerism.

After acid hydrolysis of the δ -ethyl anthranilate azopigments (1M-hydrochloric acid for 1 h at 95°C), mainly the 'vinyl' isomer was detected. The 'isovinyl' isomer was presumably destroyed during the hydrolysis since t.l.c. of the methyl esters derived after methanolysis of the same δ pigment fraction indicated the presence of the two isomers.

A similar analysis performed on the ethyl anthranilate pigments obtained from an incubation of bilirubin with UDP-glucuronic acid and liver homogenate, yielding bilirubin monoglucuronide (Black *et al.* 1970), gave, from both the α and δ fractions, the 'vinyl' and 'isovinyl' isomers.

The ratio of the isomers found in the biological material was never exactly 1:1; the 'vinyl' isomer was almost always more abundant. Even in the

δ fraction derived from the system *in vitro* the 'isovinyl'/'vinyl' isomer ratio was approx. 2:3. A similar finding has been reported by Kuenzle (1970c): in most of his azopigments derived from human bile the isomer ratio was 1:2 in favour of the 'vinyl' isomer. From these results we can conclude that monoglycosidic conjugation at one or the other carboxyethyl side chains of the bilirubin molecule occurs during the formation *in vitro* of bilirubin monoglucuronide; presumably there is no specificity of the enzyme to esterify one particular carboxyethyl side chain with D-glucuronic acid.

Kuenzle (1970c), using human bile, reported that his compound B₆, an acyl pseudoaldobiouronide (acyl 4-O- α -D-glucofuranosyl- β -D-glucopyranosiduronic acid), contained only vinylazobilirubin and no 'isovinyl' compound. We were unable, however, to isolate an azobilirubin conjugate from rat bile in which the 'isovinyl' isomer was completely lacking. However, since Kuenzle used human bile it is possible that a bilirubin conjugate with the properties of the B₆ compound was not present in the bile of the Sprague-Dawley rats from which our bilirubin conjugates were isolated.

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