The Oxidation Products of Crude Mesobilirubinogen

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Bile pigment esters were separated by ascending t.l.c. Apparently pure pigments, obtained by ferric chloride oxidation of crude mesobilirubinogen, derived from commercial bilirubin by reduction with sodium amalgam, were shown to be complex mixtures. Successive chromatography of their dimethyl esters on silica gel in methyl acetate–methyl propionate–dichloromethane–carbon tetrachloride (1:1:1:1, by vol.), ethyl methyl ketone–1,2-dichloroethane (1:2, v/v) and benzene–ethanol (100:3, v/v) revealed two major blue pigments (verdins), six major violet pigments (violins) and a red pigment (rhodin) together with numerous minor components. i-Urobilin dimethyl ester, prepared from mesobilirubinogen by dehydrogenation with aqueous iodine, was resolved into three major and at least four minor components on silica gel–kieselguhr (3:1, w/w) in benzene–ethanol (25:2, v/v). The chemical nature of these pigments was investigated by oxidation, by visible and u.v. spectroscopy, by mass spectrometry and by n.m.r. spectrometry. The evidence suggests unusual rearrangement of bilirubin during reduction leading to the formation of III α and XIII α isomers. Isomeric forms of mesobiliviolin IX α and of i-urobilin IX α may also be formed.

The significance of the differential estimation of urobilins and their chromogens in faeces has never been established with certainty because the methods of determination are unsatisfactory. The methods hitherto used have depended on polarimetric measurements and on the oxidation of urobilins with methanolic ferric chloride (Watson, Weimer & Hawkinson, 1960; Lightner, Moscovitz, Petryka, Jones & Weimer, 1969; Watson, Weimer, Petryka & Lightner, 1969). The laevorotatory stercobilin is not oxidized, the dextrorotatory d-urobilin is said to be mainly converted into a verdin, whereas optically inactive i-urobilin is mainly oxidized to a violet pigment or pigments, the chemical nature of which has never been clearly determined. Moreover, the preparation of unsaturated bile pigments such as the violins and verdins to provide standard reference compounds has involved oxidation with acidic ferric chloride in methanol (Siedel & Grams, 1940; Lemberg & Legge, 1949). For these reasons therefore the exact path of oxidation has now been investigated. Purification of the resulting violins and verdins resulting from the oxidation of crude mesobilirubinogen (the chromogen of i-urobilin) has usually involved column chromatography on magnesium oxide in chloroform–diethyl ether (Gray, Kulczycka & Nicholson, 1961) but it has been noted that much

material has remained as an immobile band at the top of the column and only a fraction migrated down the column (Watson *et al.* 1960). With efficient methods of conversion of free pigments into dimethyl esters by boron trifluoride (Cole, Chapman & Siegelman, 1967) and of dimethyl esters into free pigments the immobile pigments have been shown to be free pigments probably mixed with monomethyl esters, whereas the mobile pigments are their dimethyl esters (M. S. Stoll, unpublished work). The esters are formed by the action of methanol in the presence of hydrochloric acid during the oxidation.

This paper describes the separation by t.l.c. of the methyl esters (Chapman, Cole & Siegelman, 1967; Cole *et al.* 1967; Rudiger, 1967; Siegelman, Chapman & Cole, 1967; Troxler & Lester, 1967; Petryka & Watson, 1968) of the pigments obtained by the oxidation of crude mesobilirubinogen obtained by the sodium amalgam reduction of commercial bilirubin.

The pigment mixture has been shown to be unexpectedly complex (Scheme 1), and to consist mainly of seven violins, three verdins and a rhodin. i-Urobilin dimethyl ester prepared from commercial bilirubin has similarly been shown to consist of three major components.

Synthetic glaucobilin IX α dimethyl ester (I) and

M = methyl; E = ethyl; PM = methoxycarbonyl ethyl

glucobilin IX β dimethyl ester (II) have also been studied.

The investigation of the structures of these 16 pigments is also described. The possibility that some of the compounds were formed by association of two other compounds was eliminated by chromatography of pairs of pigments. In no case was a third pigment detected.

MATERIALS

Bilirubin was from Weddell Pharmaceuticals Ltd., London E.C.1, U.K., from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Boron trifluoride was from Cambrian Chemicals Ltd., Croydon, Surrey, U.K. All the following were from British Drug Houses Ltd., Laboratory Chemicals Division, Poole, Dorset, U.K., or from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.: acetic acid, acetone, aq. ammonia, benzene, carbon tetrachloride, chloroform, diethyl ether, ethanol, hydrochloric acid, iodine, methanol, light petroleum (b.p. 40–60°C), sodium acetate, sodium hydroxide and sulphuric acid, and were of analytical reagent grade; mercury was triple distilled; ethyl methyl ketone was special for chromatography; 1,2-dichloroethane, dichloromethane, diethylamine, ferric chloride (anhydrous), methyl acetate, methyl propionate and sodium were general-purpose reagent grade; chloroform special for spectroscopy was used for the visible and u.v. spectroscopy.

Chromatocharger and 0.1ml Hamilton syringe were from Camag Chemie-Erzeugnisse und Adsorptionstechnik A.-G., Muttentz, Switzerland. Continuous-elution t.l.c. tanks and all other t.l.c. apparatus were supplied by Shandon Scientific Co. Ltd., London N.W.10, U.K.

Synthetic glucobilins IX α and IX β dimethyl ester were gifts from Professor A. H. Jackson, Department of Organic Chemistry, University of Cardiff.

METHODS

Preparation of t.l.c. plates. The quality of the silica gel (Camag D5; Camag Chemie-Erzeugnisse und Adsorptionstechnik A.-G.) varied from batch to batch, but in most cases satisfactory results were obtained.

Quantities of silica gel and water used for silica gel plates varied according to the particular batch, and the instructions given by the manufacturer were followed. Immediately after mixing of the ingredients the slurry was shaken vigorously for 90s and then spread without delay over the plates to a depth of 0.3mm. The plates were allowed to stand for about 10min before being transferred to an oven at 110°C. They were dried for

15min in a horizontal position and then activated for a further 45min in a vertical position. The prepared plates were preserved in air-tight tins over calcium chloride and used within 7 days.

Silica gel-kieselguhr (for t.l.c. from British Drug Houses Ltd.) (3:1, w/w) plates were prepared in the same way as silica gel plates, the proportion of adsorbent to water being calculated from the manufacturers' instructions, but the final activation period in the vertical position was for 105min instead of 45min.

Preparation of sodium amalgam (3%). Sodium (3g) was added very cautiously (in fume cupboard with screen) in pea-sized portions to mercury (100g) in a beaker. It is essential to allow each portion to react before adding the next. The crude amalgam was heated gently until molten and allowed to cool in a covered container. It was then broken up and washed with cold water to remove oxidation products adhering to the surface. Finally it was washed with methanol and then ether and dried in a vacuum desiccator. The purified sodium amalgam was stored in a tightly stoppered bottle.

Preparation of crude mesobilirubinogen (Watson, 1953). Bilirubin (100mg) was reduced with freshly prepared sodium amalgam (10g) in water (1.6ml) and 0.1M-sodium hydroxide (0.4ml) by shaking for 1h. The mixture was shaken with light petroleum (300ml) under nitrogen, after the addition of acetic acid-saturated aq. sodium acetate (4:1, v/v) to bring the pH to 5.5. The petroleum was separated and the aqueous layer shaken twice more with petroleum (2 $\times$ 150ml). The combined petroleum extract was washed twice with water (2 $\times$ 20ml) and filtered.

The petroleum solution of crude mesobilirubinogen may now be used either to prepare i-urobilin or crude mesobiliviolin and glucobilin.

Preparation of crude mesobiliviolin and glucobilin (Gray et al. 1961). The petroleum extract of crude mesobilirubinogen from the reduction of bilirubin (100mg) was evaporated to dryness. The residue was dissolved in methanol (10ml) and 20% (w/v) ferric chloride in 12M-hydrochloric acid (1ml) was added. The mixture was boiled under reflux for 20min, cooled, diluted with water (35ml) and extracted repeatedly with diethyl ether until no more colour passed into the ether. The ethereal solution was then washed with water until no further pigment passed into the aqueous layer. The aqueous solutions contained no violin or verdin and were discarded. The ethereal solution was then exhaustively extracted with 2.8M-hydrochloric acid, after which little colour remained in the ether, which was discarded. The acid solution was exhaustively extracted with chloroform and the combined chloroform extract washed with water, dried by filtration and evaporated to dryness. The crude pigment mixture at this stage consisted of a mixture of free and methyl esterified pigments.

Preparation of i-urobilin (modification of the method of

Watson, 1953). The petroleum extract of crude mesobilirubinogen from the reduction of bilirubin (100mg) was shaken with 50 ml portions of an aqueous solution of iodine [prepared by adding ethanolic 1% (w/v) iodine (4.5 ml) to water (400 ml)] and then repeatedly with water until no further colour passed into the aqueous layer. The combined aqueous extract was acidified with hydrochloric acid (final concn. 1M) and the acid solution exhaustively extracted with chloroform. The chloroform solution was dried by filtration and evaporated to a volume of 1–2 ml. Hot acetone (3.5 ml) was added, with stirring, to the hot chloroform extract. The urobilin, which crystallized from solution, was removed by centrifugation and recrystallized from chloroform–acetone to constant extinction coefficient [ϵ 69.9×10^3 at 499 nm ($\lambda_{\text{max.}}$) in chloroform].

Methyl esterification of the bile pigments (Cole et al. 1967). The pigment was dissolved in methanol (10 ml), and 15% (w/w) boron trifluoride in methanol (5 ml) was added. The mixture was boiled under reflux for 3 min, cooled and poured into chloroform (20 ml). The mixture was washed with water (5×25 ml) until no further change in colour occurred. The chloroform solution was dried by filtration and evaporated to dryness.

Saponification of pigment dimethyl esters to free pigments (modification of method of Muir & Neuberger, 1949). The pigment ester was dissolved in 50% (v/v) sulphuric acid (1 ml) under nitrogen and kept at room temperature in the dark for 2 h. The solution was then poured into water (10 ml), and 12M-hydrochloric acid (1 ml) added. The remainder of the extraction depended on the pigment.

(a) Verdins. The solution was exhaustively extracted with ether. The ethereal solution was extracted with *m*-ammonia, after which the aqueous solution was acidified with hydrochloric acid and exhaustively extracted with ether. The ethereal solution was washed with water, dried by filtration and evaporated to dryness. The residue was free pigment.

(b) Violins. The solution was exhaustively extracted with chloroform. The chloroform solution was extracted with *m*-ammonia, after which the aqueous solution was acidified with hydrochloric acid and exhaustively extracted with chloroform. The chloroform solution was washed with water, dried by filtration and evaporated to dryness. The residue was free pigment.

(c) Urobilins. The solution was exhaustively extracted with chloroform. The chloroform solution was exhaustively extracted with water, after which the aqueous solution was acidified with hydrochloric acid and exhaustively extracted with chloroform. The chloroform solution was dried by filtration and evaporated to dryness. The residue was urobilin hydrochloride, which was crystallized from chloroform–acetone.

T.l.c. of crude mesobiliviolin and glaucobilin dimethyl esters. The chromatographic systems used were developed from that used to isolate phycoerythrobilin dimethyl ester (Chapman et al. 1967). The system used was silica gel developed with methyl acetate–carbon tetrachloride (2:1, v/v). Solvent vapour in the development tanks was allowed to reach equilibrium before development was begun, but no time for equilibration between plates and solvent vapour was allowed. For the development of the new systems 5 cm $\times$ 20 cm plates were chromatographed in cylindrical jars. The pigment was applied in chloroform

as a spot with a glass capillary. Of several types of silica gel investigated Camag D5 gave by far the highest resolution. Variations in the polar component of the solvent system were made and, with the two series of esters, methyl acetate, methyl propionate, methyl *n*-butyrate and methyl acetate, ethyl acetate, *n*-propyl acetate, the highest R_F value was obtained with methyl acetate, but optimum separation was obtained with a mixture of methyl acetate and methyl propionate. Variations in the non-polar component were also made. A mixture of carbon tetrachloride–dichloromethane (1:1, v/v) and methyl acetate–methyl propionate (1:1, v/v) gave the best overall separation. The proportion of the former mixture to the latter could be varied according to the activity of the silica gel and the particular separation required. The best overall separation of the pigments in the mixture was obtained with carbon tetrachloride–dichloromethane–methyl acetate–methyl propionate (1:1:1:1, by vol.) (solvent system I). The solvent front was allowed to reach the top of the plate, which was then removed from the tanks, dried and, without elution, again developed with the same solvent system.

Solvent systems with 1,2-dichloroethane gave a markedly different type of separation and the best overall system was ethyl methyl ketone–1,2-dichloroethane (1:2, v/v) (solvent system II) run twice. From numerous solvent systems investigated only those with 1,2-dichloroethane as the non-polar component gave a basically different separation from solvent system I. Diethyl esters prepared by using ethanolic boron trifluoride migrated with higher R_F than dimethyl esters, from which they could be easily separated, but appeared to offer no advantage. Similarly, di-*n*-propyl esters migrated with higher R_F than diethyl esters.

To establish the total number of pigments separable with solvent systems I and II, two-dimensional chromatograms were run on 20 cm $\times$ 20 cm plates with the two solvent systems with the mixture of pigment esters applied as a concentrated spot in one corner of the plate. This revealed six prominent violet spots and numerous faint ones, together with two blue spots of comparable abundance (Fig. 1). There were also faint spots of several other colours. Visible spectroscopy of the pigments

Fig. 1. Two-dimensional chromatogram on silica gel of crude mesobiliviolin and glaucobilin dimethyl esters (only the major spots are shown).

indicated that violins 3 and 5 contained components absorbing at about 500–520 nm. The system benzene-ethanol (25:2, v/v) (Petryka & Watson, 1968) on silica gel partially resolved these pigments into red and violet components. Further work resulted in complete separation of violins 3 and 5 each into a red component (rhodin) and a violin by chromatography on silica gel in benzene-ethanol (100:3, v/v) (solvent system III) in a continuous elution tank, the running time being about 6 h. The chromatographic characteristics of this system are similar to those of solvent system I, but it shows some additional resolution, particularly as described above. Each violin was finally purified by chromatography in this system, whereupon minor components were removed from some violins, particularly from violin 1 (Fig. 2). However, the separations finally obtained can only be achieved by the use of all three solvent systems in the manner described. Only the nine major pigments have so far been investigated.

For preparative-scale isolation the technique adopted was to chromatograph the crude pigment ester in solvent system I, remove the bands of interest and rechromatograph these in solvent system II. Finally violins 1, 4, 2a, 3 and 5 were chromatographed in solvent system III. In this way the nine pigments could be isolated on a comparatively large scale.

Crude pigment ester prepared from bilirubin (100 mg) was chromatographed on five 20 cm × 20 cm plates in a large tank. The pigment was dissolved in chloroform (0.4 ml) and one-fifth of this solution was applied to each plate with a Chromatocharger (see the Materials section) in a streak about 18 cm long. The chromatographic pattern obtained is shown in Fig. 3. In this preparation the loading (about 5 mg) of the plates is near the limit possible without loss of resolution.

The five main bands indicated in Fig. 3 were removed from the plates by suction and the pigments were eluted from the silica gel by washing with acetone until no further colour could be extracted. The acetone solutions of the five pigments were then evaporated to dryness.

For chromatography in solvent system II bands 2 and 5 were run together on five 20 cm × 20 cm plates; bands 1

and 4 were run together on five 20 cm × 20 cm plates and band 3 was run on two 20 cm × 20 cm plates. It was possible to chromatograph certain pigments together because it was ascertained that no interference occurred; this procedure saved time and materials.

In solvent system II bands 1 and 5 remained essentially unique; band 2 resolved into three violet bands of comparable abundance, and with very close R_f values, and band 4 resolved into a blue band and a minor olive-green band (not isolated) with the same R_f value as band 5. In all cases numerous very faint bands were observed on rechromatographing any pigments. These are believed to be decomposition products. The eight pigments thus obtained were extracted from the plates as before.

For final chromatography in solvent system III, 0.5 mm silica gel plates were used in continuous elution tanks. Violin 1 was chromatographed on two plates and violins 4, 2a, 3 and 5 on one plate each. The running time for each was about 6 h. From these the major violin bands and the two main red bands were recovered in the usual way. Chromatography in all three systems showed that the two red bands were the same. This therefore was called the rhodin.

Six more violet pigments have now been isolated as minor components from the oxidation products of the sodium amalgam reduction product obtained from 200 mg of bilirubin and in addition oxidation experiments described below resulted in the isolation of a third verdin (verdin 3) and a stable component of violin 3 (violin 3 stable).

T.l.c. of i-urobilin dimethyl ester. A different system was used for the chromatography of i-urobilin dimethyl ester. The system was a development of that used by Petryka & Watson (1968). Plates of silica gel-kieselguhr (3:1, w/w) were used and the solvent system was benzene-ethanol (25:2, v/v) run twice. Three main bands were observed (Fig. 4).

For preparative-scale chromatography i-urobilin dimethyl ester from bilirubin (100 mg) was chromatographed on five 20 cm × 20 cm plates. The pigments were removed by extraction into acetone.

Oxidation. The violins can readily be dehydrogenated by ferric chloride to the corresponding verdin, e.g. mesobiliviolin IX α dimethyl ester (III) gives glaucobilin

Fig. 2. Chromatogram of bile pigment dimethyl esters on silica gel in solvent system III. Continuous elution was carried out for about 6 h.

Fig. 3. Chromatogram on silica gel in solvent system I of crude mesobiliviolin and glaucobilin dimethyl esters (only the prominent bands are shown).

IX α dimethyl ester. Similarly i-urobilin IX α dimethyl ester (IV) is dehydrogenated to mesobiliviolin IX α dimethyl ester. This technique has been used to investigate the relationship between the new urobilins, violins and verdins.

The pigment ester was dissolved in methanol (2ml) and 0.2ml of 20% (w/v) ferric chloride in 12M-hydrochloric acid added. The mixture was boiled under reflux for the time stated (15min, 1h or 2h), poured into chloroform (5ml) and washed with water (3 $\times$ 10ml). The chloroform solution was dried by filtration and evaporation to dryness.

Tests for interconversion. The 14 pigments isolated were each rechromatographed in appropriate solvent systems and shown to be unique, thus proving that none was an artifact of the chromatographic procedure. The possibility that some pigments were interconvertible was also studied by subjecting them again to conditions similar to those by which they were produced: (a) treatment with methanolic ferric chloride; (b) treatment with methanolic boron trifluoride; (c) saponification and re-esterification; (d) treatment of urobilins with acid.

The urobilin ester was dissolved in chloroform and the solution shaken with 0.1M-hydrochloric acid. The chloroform solution was then shaken with water three times, dried by filtration and evaporated to dryness.

The control urobilin esters used in this experiment were maintained in contact with diethylamine and chromatograms were developed in solvent systems to which was added 1% (v/v) of diethylamine, to provide a proton-free environment.

T.l.c. T.l.c. was used to analyse the products of reaction in the oxidation and interconversion experiments. T.l.c. was carried out on silica gel and unless otherwise stated all chromatography was carried out in duplicate in solvent systems I and II. Comparisons were made with internal

standards since absolute R_f values may vary slightly in different positions on the same plate.

Visible and u.v. spectroscopy. Spectra were recorded on a Unicam SP.800 automatic spectrophotometer, in chloroform solution, between 315 and 700nm. The spectra of similar compounds were recorded at comparable concentrations for the closest comparison.

Mass spectrometry. Mass spectra were recorded by Mr D. W. Carter of the University of London Mass Spectrometry Service at the School of Pharmacy using an A.E.I. MS902 double-focusing mass spectrometer. The temperature of the heated inlet to the mass spectrometer was 250°C, the samples were inserted on a direct probe and the electron impact potential was 70 V. All mass spectra were corrected for varying total ion intensity and normalized histograms were prepared from these data. Except where stated, the spectra of compounds in each class of pigment showed similar fragment ions but of different relative intensities. However, similar spectra may seem more different than they really are because, in the histograms presented, ions of very low intensity are not shown although the lowest percentage intensity included is indicated in the legend to each figure.

N.m.r. spectrometry. The proton-magnetic-resonance (p.m.r.) spectra of the five verdins in deuteriochloroform were recorded by Mr P. N. Jenkins of Imperial College, London, using a Varian HA-100 100MHz spectrometer, and those of violins 1, 2b, 4 and 5, which were expected to be more complex, by Dr D. K. Beconsall of Imperial Chemical Industries Ltd. (Runcorn, Cheshire, U.K.) with the more sensitive Varian HR-220 220MHz machine. The spectrum of violin 2a could not be obtained.

In obtaining the spectra of the verdins sufficient verdins 1 and 2 were available to give adequate spectra in a single scan, but the small quantities of the other three verdins necessitated the use of a computer of average transients to secure spectra from nine separate scans in each case. Despite instrumental interference and impurities present there was sufficient detail to permit interpretation. In verdins 2 and 3 spectrum expansions were performed to show the separation of signals separated by only 0.02 p.p.m. An approximate spectrum integration was possible with verdin 1 only.

In obtaining the spectra of the violins a computer of average transients was used and spectra were obtained from the accumulation of ten separate scans in each case; a 60-scan accumulation of violin 2b was also obtained. Spectrum integrations were obtained with violins 1 and 2b only.

Some difficulty was experienced in determining τ values in the spectra obtained by use of a computer of average transients and better correlations between spectra were obtained if the chloroform signal, known to vary very little in position, was regarded as an internal reference at 2.80 τ as found in both the single-scan spectra, which

Fig. 4. Chromatogram on silica gel-kieselguhr (3:1, w/w) in benzene-ethanol (25:2, v/v) of i-urobilin dimethyl ester.

(III)

(IV)

contained good tetramethylsilane reference signals. The assignments were made both from fundamental considerations of proton environment and from mutual comparisons and comparisons with the published spectra of similar compounds (Chapman *et al.* 1967; Cole *et al.* 1967; Crespi, Boucher, Norman, Katz & Dougherty, 1967; Siegelman, Chapman & Cole, 1968).

RESULTS AND DISCUSSION

Verdins

Further esterification, oxidation with methanolic ferric chloride and saponification and re-esterification resulted in no changes attributable to interconversion or further oxidation. These experiments were performed on the two most abundant verdins, 1 and 2; no violet pigments (purpurins) were formed on further oxidation for 15 min.

T.l.c. The verdins all migrated with R_f values about 0.7. They were clearly separated and glaucobilin IX α dimethyl ester used as an internal standard had the same mobility as verdin 2. Verdin 1 was slightly slower, verdin 3 slightly faster.

Visible and u.v. spectroscopy. Table 1 shows the spectra of the three verdins and glaucobilins IX α and IX β . The spectra of verdin 2 and of glaucobilin IX α dimethyl ester were indistinguishable and differed slightly from those of verdins 1 and 3; that of glaucobilin IX β dimethyl ester differed considerably from the other four.

Mass spectrometry. Fig. 5 shows the mass spectra of the five verdins; the m/e values of only those ions of particular interest are given. The spectra of verdins 1 and 2 and glaucobilin IX α dimethyl ester were very similar, there being only minor differences in the relative intensities of some ions. The spectrum of verdin 3 was also similar but showed a greater abundance of ions of low molecular weight. All show the base peak at m/e 614, which is also the parent ion. This corresponds to the formula $C_{35}H_{42}N_4O_6$ and shows that the compounds are isomeric. All the ions shown in the spectrum of verdin 3 were also present in the spectra of verdins 1 and 2 and glaucobilin IX α dimethyl ester.

The spectrum of glaucobilin IX β dimethyl ester was very different from those of the other verdins,

showing ions at m/e 360 and 254 representing fragmentation across the central methene bridge. It differed also from that described by Jackson & Kenner (1968); the latter showed extensive fragmentation and the base peak was a low-molecular-weight ion, the parent ion at m/e 614 being a minor peak; this is because of differences in the decomposition of heat-labile materials due to differences in the temperature of the heated inlet and the time spent in the inlet by the sample.

Interpretation of t.l.c., spectroscopy and mass spectrometry. The great similarity in chromatographic behaviour, in the visible and u.v. spectra and in the mass spectra of verdins 1, 2 and 3, suggests that the compounds are very closely related isomers, closer than the IX α and IX β isomers.

Such isomerism may be (a) *cis-trans* isomerism about bridge double bonds, (b) steric interaction in the bulky methoxycarbonyl ethyl or carboxyethyl side chains, which may produce a pair of enantiomers or, if a similar interaction occurs between the end-ring carbonyl groups, a pair of diastereoisomers separable by t.l.c., or (c) isomerism due to different orders of β -substituents.

(a) That i-urobilin IX α dimethyl ester is oxidized to verdin 2 only (see below) eliminates the possibility of *cis-trans* isomerism about the outer bridge double bonds since all possible forms would be generated in this reaction.

Glaucobilin dimethyl ester from mesobilirubin prepared from bilirubin under alkaline conditions (Fischer & Orth, 1943) was a mixture of all three verdins. However, *cis-trans* isomerism about the central bridge double bond would be expected to account for only two verdins. It is not yet known whether mesobilirubin is unique, and gives rise to the three verdins on oxidation, or whether mesobilirubin consists of three compounds each corresponding to one verdin.

(b) The possibility of steric interaction in the bulky methoxycarbonyl ethyl side chains can be discounted since saponification and re-esterification of verdins 1 and 2 causes no interconversion. Steric interaction between the carboxyethyl side chains, however, remains a possibility but would be expected to account for only two verdins.

Table 1. *Visible and u.v. spectra, in chloroform, of verdin dimethyl esters*

Pigment	U.v. (nm)	Visible (nm)		$10^{-3} \times \epsilon_{u.v.}$	$10^{-3} \times \epsilon_{vis.}$	$\epsilon_{u.v.}/\epsilon_{vis.}$
	$\lambda_{max.}$	$\lambda_{max.}$				
Verdin 1	367.5	634		41.4	11.8	3.51
Verdin 2	367.5	631		44.3	12.45	3.56
Verdin 3	367.5	630		—	—	3.57
Glaucobilin IX α dimethyl ester	367.5	631		—	—	3.55
Glaucobilin IX β dimethyl ester	369	643		—	—	3.42

Fig. 5. Normalized histograms of the mass spectra of verdins (all ions of relative intensity $\geq 4\%$ are shown).

(c) Rearrangement of the β -side chains could arise by fission of the bilirubin molecule at the centre bridge during alkaline reduction with subsequent recombination of half-molecule fragments or attack of such fragments on intact bilirubin molecules with displacement of new half-molecule fragments. Combination of similar half-molecules would give rise to symmetrical pigments of the $\text{III}\alpha$ and $\text{XIII}\alpha$ series; other rearrangements are

unlikely because of the terminal carbonyl groups in rings A and D. Thus $\text{III}\alpha$, $\text{IX}\alpha$ and $\text{XIII}\alpha$ pigments might be expected in products obtained by alkaline reduction of bilirubin. The great similarity between these three series of pigments is consistent with the similarity of verdins 1, 2 and 3. Support is provided by the t.l.c. separation of synthetic glaucobilin $\text{III}\alpha$ (V), $\text{IX}\alpha$ and $\text{XIII}\alpha$ (VI) dimethyl esters (H. Pleininger, personal communication). The $\text{III}\alpha$

pigment had the highest R_F value and the XIII α the lowest; the separation is therefore as found for verdins 1, 2 and 3.

The mass spectra of all the verdins (except glaucobilin IX β dimethyl ester) and violins contain ions at about m/e 315 and 302 due to fission of the molecules at the central bridge, thus showing that the total mass of β -substituents is equal in the two halves of the molecule. The mass spectra of all the violins show fragments formed by loss of one or other of the end rings containing one methyl and one ethyl β -substituent. Thus only isomers in which β -substituents on any individual rings are interchanged are possible. It is extremely difficult to postulate any mechanism whereby interchange of substituents on the centre rings may occur and thus only the III α and XIII α isomers are probable.

P.m.r. spectrometry. Fig. 6 shows the p.m.r. spectra of the five verdins. Those of the dimethyl esters of verdins 1, 2 and 3 may be interpreted on the assumption that these pigments are glaucobilins XIII α , IX α and III α respectively. The IX α pigment thus differs from the XIII α pigment in the interposition of the methyl and ethyl groups of one end ring. In glaucobilin III α , the methyl and ethyl groups have been interposed in the second end ring. As expected therefore the spectra of verdins 1 and 3 both show greater symmetry than that of verdin 2.

The signals in the range 7.9–8.3 τ are within the region characteristic for methyl groups attached to double bonds (Jackman, 1959) and these therefore represent β -methyl substituents. The β -methyl group of the two centre rings B and C share an almost identical environment in all three pigments. Thus in all three verdins there are signals at 7.97 τ due to six protons, i.e. two equivalent methyl groups, which must be the methyl groups of rings B and C. In the XIII α compound the environment of the methyl groups of the end rings A and D will be virtually identical, but different from the environment of the two similar corresponding methyl groups in the III α compound. In verdin 1 there is a singlet at 8.225 τ due to six protons from the methyl groups in the outer positions of rings A and D. In verdin 3 the singlet is at 7.95 τ and due to six protons corresponding to the inner methyl groups of rings A and D. In the IX α compound, on the other hand, the environment of the methyl group in ring A will be that of the same group in the XIII α compound. In verdin 2 two separate singlets each of intensity corresponding to three protons are at 8.225 τ and 7.95 τ corresponding to the methyl groups in rings A and D respectively. The methyl groups in rings A and D in verdin 3 and that in ring D of verdin 2 are responsible for the singlets at 7.95 τ , which is close to but significantly different from the singlet given by all

compounds at 7.97 τ due to the unchanging position of the methyl groups in rings B and C. This is consistent with the interpretation that the environments of these groups are very similar.

Similarly the environment of the methyl components of the two ethyl groups will be virtually identical in an XIII α structure; in the IX α structure that of the methyl component of one of the ethyl groups will have changed and in the III α structure that of the methyl component of the other ethyl group will also have changed similarly. In verdin 1 there was a triplet signal at 8.82 τ due to six protons, presumably from the methyl components of the ethyl groups in the inner positions of rings A and D. In verdin 2 there are two overlapping triplets at 8.82 τ and 8.955 τ each due to three protons and accounted for by the methyl component of the ethyl group in ring A and that in ring D respectively. In verdin 3 the triplet at 8.955 τ is due to the methyl component of the ethyl group in the outer position of rings A and D.

Verdin 1 thus differs from verdin 2 in that the position of the methyl and ethyl substituents in one end ring are interposed and verdin 3 differs from verdin 2 in that the positions of the methyl and ethyl groups in the second end ring have been interposed, i.e. it differs from verdin 1 in that the β -substituents in both end rings have been interposed.

The spectra of verdins 1, 2 and 3 also show signals due to other protons in the molecules; a singlet at about 3.32 τ (corresponding to one proton) is due to the b bridge proton and that at about 4.16 τ (corresponding to two protons) to the a and c bridge protons, which are clearly in almost equivalent environments. The singlet at about 6.39 τ (corresponding to six protons) is due to the methoxyl groups of the two almost equivalent methoxycarbonyl ethyl substituents. Triplet signals at about 7.13 τ and 7.51 τ (corresponding to four protons each) are due respectively to the β and α pairs of almost equivalent methylene groups in the methoxycarbonyl ethyl substituents, and ill-defined absorption in the same region to the methylene groups of the β -ethyl substituents.

The spectrum of glaucobilin IX β dimethyl ester reflects the highly asymmetric order of β -substituents, such that most are in sufficiently different environments to give separate p.m.r. signals. Thus, as well as a signal at 3.39 τ from the b bridge olefinic proton, there are signals at 4.07 τ (corresponding to one proton) and 4.18 τ (corresponding to one proton) due to the a and c bridge olefinic protons respectively. Signals at 6.33 τ and 6.37 τ are due to the methoxyl groups of the β -methoxycarbonyl ethyl substituents. The signal at 7.89 τ is due to the two β -methyl substituents of rings B and C, that at 7.93 τ to the β -methyl substituent in ring D and

Fig. 6. P.m.r. spectra of verdins, in deuteriochloroform, at 100MHz. TMS, tetramethylsilane.

that at 8.17 τ to the β -methyl substituent in ring A. The overlapping triplets at 8.92 τ and 8.97 τ are due to the methyl components of the β -ethyl substituents of the C and D rings respectively. These values are in fair agreement with those of Jackson, Kenner & Smith (1966). The precise assignments for glaucobilin IX β dimethyl ester made by analogy with the spectra of verdins 1, 2 and 3 must be regarded as tentative.

The spectrum of glaucobilin IX α dimethyl ester in $[\text{}^2\text{H}_5]\text{pyridine}$ recorded by Cole *et al.* (1967) did not show the same resolution of the signals of the β -methyl substituents or of the methyl components

of the β -ethyl substituents but this must be a solvent effect (Bible, 1965).

Violins

The two most abundant violins, 1 and 2b, were tested for possible interconversion and none was found; verdins were the sole products of their oxidation.

Oxidation. On 15 min oxidation by methanolic ferric chloride violin 1 gave verdin 1, violins 2a and 2b gave verdin 2 and violin 3 gave verdin 3, this last in smaller yield and the possibility that

Fig. 7. Normalized histograms of mass spectra of violins [all ions of relative intensity $\geq 5\%$ are shown, except violin 3 (stable), where only those $\geq 10\%$ are shown].

this was due to a stable component in violin 3 was investigated. When violin 3 was oxidized for 2h with methanolic ferric chloride verdin 3 and a residual stable violin referred to as violin 3 (stable) were isolated by t.l.c. on silica gel in benzene-ethanol (100:3, v/v). Violins 4 and 5 were stable to oxidation.

Visible and u.v. spectroscopy. The u.v. maxima of the seven violins ranged from 327 to 328.5nm, the visible maxima from 563 to 566nm and the ratios of the intensities of the visible and u.v. maxima were between 1.26 and 1.32. The difference of about 3nm in the visible maxima of violins 2a and 2b, however, has been consistently recorded

and undoubtedly reflects a different order of side chains attached to the chromophore, an effect observed in the verdins.

The formation of verdins 1 and 3 by the oxidation of violins 1 and 3 shows that clearly violin 1 is a XIII α pigment whereas violin 3 is mainly composed of a III α pigment. Similarly the formation of verdin 2 from violins 2a and 2b shows that these must be IX α compounds.

An explanation for the existence of two IX α violins (violins 2a and 2b) is possible. The violin chromophore, unlike that of verdins, is asymmetric. It therefore follows that violins with a symmetrical order of side chains, e.g. III α and XIII α , can exist in only one form, but violins with an unsymmetrical order of side chains, e.g. IX α , can exist in two forms. Thus it is suggested that violins 2a and 2b are mesobiliviolin IX α dimethyl ester and isomesobiliviolin IX α dimethyl ester (VII) (Siedel & Möller, 1940). This is consistent also with the visible spectra of the compounds.

The u.v. and visible spectra of the violins are extremely similar, thus supporting the very similar structures proposed.

Mass spectrometry. Fig. 7 shows the mass spectra of the seven violins. The spectra of violins 2a and 2b were extremely similar, the only minor differences being in the relative intensity of some ions. The spectrum of violin 1 is also very similar but the relative intensity of the base peak is smaller.

The spectra of violins 4 and 5 were extremely similar to one another, but differed significantly from those described above. The spectrum of violin 3 (stable) was similar to those of violins 4 and 5, but the base peak was of smaller relative intensity. The spectrum of violin 3 showed features of both the labile violins and the stable violins.

These mass spectra fall into two groups, that of the labile violins with a base peak at m/e 492 and that of the stable violins with a base peak at m/e 493 corresponding to the fragment ion (VIII) or an isomer of it.

All the labile violins exhibit three ions in the

parent region at m/e 614, 616 and 618. As observed by Watson *et al.* (1969) for the urobilins, the pigments undergo a redox reaction in the heated inlet to the mass spectrometer producing ions of higher and lower mass. The true parent ion at m/e 616 produces a very stable ion at m/e 614 and a more labile ion at m/e 618.

The spectra of the stable violins exhibit similar complex parent ions, but the ion at m/e 618 is much more intense, thus suggesting that the pigments contain an hydrogenated end ring outside the chromophore. The reason for the base peak appearing at m/e 493 instead of m/e 492 is not known, but appears to be characteristic of stable violins. The parent ions and base peak of violin 3 give support for the evidence provided by oxidation that this pigment consists of labile and stable components.

P.m.r. spectrometry. The p.m.r. spectra of the violins are shown in Fig. 8. The spectra of violins 1, 2b, 4 and 5 can be interpreted by accepting that violin 1 is mesobiliviolin XIII α dimethyl ester (IX), as proved by its oxidation to verdin 1, and that violin 2b must have the IX α structure, as proved by its oxidation to verdin 2. There are signals in the range approx. 8.0–8.5 τ due to β -methyl substituents. Because of the greater asymmetry of the violins, compared with the verdins, each β -methyl substituent is in a sufficiently different environment to give a distinct p.m.r. singlet. Violins 1 and 2b, known to contain four β -methyl substituents attached to double bonds, each give four corresponding singlets. Violins 4 and 5, because of their stability to ferric chloride oxidation and their mass spectra, are believed to contain an unconjugated hydrogenated end ring, and in accordance with this each gives only three singlets corresponding to three β -methyl groups attached to double bonds, i.e. the β -methyl substituents of the chromophore in each compound; singlets at 7.98, 8.06 and 8.10 τ (each equivalent to three protons) in the spectrum of violin 2b and singlets corresponding to within 0.03 p.p.m. of these in the

Fig. 8. P.m.r. spectra of violins, in deuteriochloroform, at 220 MHz.

spectra of violins 1, 4 and 5 must correspond to β -methyl substituents in very similar environments in each compound, i.e. each violin must contain the same chromophore (X) as mesobiliviolin XIII α .

Further supporting this, the spectra of both violins 1 and 2b show a triplet at about 8.84τ (corresponding to three protons) due to the methyl component of the β -ethyl group in the inner position of ring A. The corresponding signals in the spectra of violins 4 and 5, however, are obscured as explained below.

In violin 1 there is a singlet at 8.34τ (corresponding to three protons) due to the β -methyl

substituent in the outer position of ring D and in violin 2b the singlet is at 8.17τ corresponding to the β -methyl substituent in the inner position of ring D. Similarly, in violin 1 there is a triplet at 8.97τ (corresponding to three protons) due to the methyl component of the β -ethyl substituent in the inner position of ring D and in violin 2b the triplet is at 9.22τ corresponding to the methyl component of the β -ethyl substituent in the outer position of ring D. In comparing the spectra of violins 1 and 2b therefore similar shifts of signals, due to the interposition of β -substituents in the D-ring, are observed as in the spectra of verdins 1 and 2.

Violin 2b therefore must be mesobiliviolin IX α dimethyl ester and not isomesobiliviolin IX α dimethyl ester.

Signals due to the methyl components of the β -ethyl groups in violins 4 and 5 are more difficult to interpret. The signal around 8.84 τ due to the A ring ethyl β -substituent is obscured by its proximity to the doublet presumably present in the same region, due to the β -methyl substituent of the hydrogenated ring D (Chapman *et al.* 1967). The triplet at around 9.21 τ seen in both spectra must be due to the methyl component of the β -ethyl substituent in ring D. Presumably the three-dimensional environment of such a group is virtually identical in both the inner and outer positions.

Since violins 4 and 5 contain the part structure (X) and an unconjugated hydrogenated end ring there are only two possible XIII α , IX α or III α structures, i.e. dihydromesobiliviolin IX α dimethyl ester (XI) and dihydromesobiliviolin XIII α dimethyl ester (XII), and since violin 4 arises by oxidation of a hydrogenated component present in i-urobilin IX α (see the next section) it probably has the IX α structure, which is in accordance with the results of Petryka *et al.* (1968), and on this basis violin 5 would have the XIII α structure. The p.m.r. data so far available, however, do not permit this distinction to be made.

The p.m.r. spectra of all the violins studied have signals within 0.03 p.p.m. at about 4.12 τ and 3.30 τ due to the a and b bridge olefinic protons respectively. As expected there is no signal corresponding to the c bridge protons in this region because in the violins this bridge is hydrogenated. All spectra also have signals within 0.04 p.p.m. at about 6.39 τ due to methoxyl groups of the methoxycarbonyl ethyl substituents, and in violins 1 and 2 this signal is partially separated, since the environment of the

two groups is somewhat different. Signals within 0.04 p.p.m. at about 7.14 τ and 7.53 τ are due respectively to the β and α pairs of almost equivalent methylene groups in the methoxycarbonyl-ethyl substituents. In the 60-scan spectrum of violin 2b signals at 5.73 τ and 6.94 τ , due to the c bridge methylene group and the ring D α hydrogen atom respectively, can be seen.

However, although inspection of the conventionally written formulae for linear bile-pigment structures would suggest differences in environment of the β -substituents, true differences are likely to be dependent on the three-dimensional helical structure (Moscowitz, Kraeger, Kay, Skaves & Bruckenstein, 1964).

Structures of violins 2a and 3. Since violin 2b has been identified as mesobiliviolin IX α dimethyl ester, violin 2a most probably is isomesobiliviolin IX α dimethyl ester. By analogy with violin 1 it is most probable that the labile component of violin 3 is mesobiliviolin III α dimethyl ester (XIII).

The structure of violin 3 (stable) can only be surmised, but it is probably either dihydroisomesobiliviolin IX α dimethyl ester (XIV) or dihydromesobiliviolin III α dimethyl ester (XV), since these are the only two pigments in the series being considered that have not been identified, and they would be consistent with the data so far available on the pigment.

Rhodin

The small quantities of rhodin isolated during the purification of violins 3 and 5 have been little studied. The chromatographic behaviour, visible spectrum and mass spectrum allow a probable part structure for the compound to be proposed.

The urobilins, violins and verdins all have one

(XI)

(XII)

(XIII)

(XIV)

(XV)

(XVI)

basic pyrrolenine nitrogen atom within the chromophore. The base strength decreases in the order shown, as found by spectrophotometric titration studies (Gray *et al.* 1961). It appears therefore that the base strength decreases with increasing length of conjugation and the extent of this can, in turn, be assessed from the position of the visible absorption maximum. Thus an increase in the length of conjugation produces a bathochromic shift in the absorption maximum. On this basis, the single absorption maximum of the rhodin at 501.5nm, being between that of the urobilins and the violins, suggests a chromophore whose length is similarly intermediate. In chromatography, verdins have the highest R_F values and urobilins the least and so R_F values increase with decreasing base strength. Thus the expected R_F value for the rhodin, if the molecule contains a pyrrolenine nitrogen atom within the chromophore, would be between the urobilins and the violins. In fact, however, it migrates with the violins, which indicates a less basic character than predicted and an interpretation of this is that it contains no pyrrolenine nitrogen atom.

The mass spectrum (Fig. 9) is intermediate between that of the violins and the urobilins. The base peak is at m/e 494, corresponding to loss of an end ring, and characteristic of urobilins suggesting that the rhodin is in the same oxidation state as the corresponding urobilin. However, the peak at m/e 371, corresponding to loss of the second end ring, so prominent in the spectra of urobilins and absent from that of the violins, is a minor peak in the rhodin. This shows that in the rhodin one end bridge is saturated and the other only partially saturated, whereas in the urobilins both bridges are saturated, and in the violins one is saturated and the other fully unsaturated. That rhodins are close isomers of urobilins is suggested by the work of Ó hEocha, Ó Carra & Carroll (1964), who showed that the latter were readily isomerized to the former by strong acid. A part structure consistent

with these data is (XVI), the order of β -substituents in the terminal rings not being known nor the degree of hydrogenation of ring A.

Urobilins

Oxidation. The two major pigments (urobilins 2a and 2b) were each oxidized in methanolic ferric chloride for 15 and 60min and the products were examined by t.l.c. The products from each pigment were indistinguishable and consisted of a single verdin and three violins, two of which were labile. Internal standards showed that the verdin was verdin 2 (glucobilin IX α dimethyl ester). The labile and one stable violins were identified as violins 2a, 2b and 4. Consequently then the two urobilins are believed to be isomeric forms of

Fig. 9. Normalized histogram of the mass spectrum of the rhodin (all ions of relative intensity $\geq 5\%$ are shown).

Fig. 10. Normalized histograms of the mass spectra of urobilins (all ions of relative intensity $\geq 5\%$ are shown).

i-urobilin IX α dimethyl ester mixed with ring D dihydrogenated products. Similar oxidation of urobilin 1, which was only available in small quantities, gave unidentified violin but no verdin.

Possible interconversion and properties of unesterified pigment hydrochlorides. The following experiments were not performed on urobilin 1 because only very small quantities were available. The possibility that urobilins 2a and 2b were interconvertible in acid was studied. Each was treated with hydrochloric acid and after neutralization the products were examined by t.l.c. on silica kieselguhr (3:1, w/w) in benzene-ethanol-diethylamine (100:8:1, by vol.). No interconversion was observed.

The known unesterified urobilin hydrochlorides crystallize well from chloroform-acetone. The behaviour of urobilins 2a and 2b on crystallization has therefore been compared. Urobilins 2a and 2b were saponified and the hydrochlorides of the free unesterified pigments isolated. Free urobilin 2b hydrochloride crystallized with great ease from chloroform or chloroform-acetone, but free urobilin 2a hydrochloride was much more soluble and crystallized only with great difficulty. These two urobilin hydrochlorides were reconverted into the dimethyl esters and the products examined by t.l.c. in benzene-ethanol (25:2, v/v). Some interconversion of the two pigments had occurred, but urobilin 2b was the more stable.

Visible and u.v. spectroscopy. Urobilins 1, 2a and 2b showed single maxima at 456, 451 and 452nm respectively, indicating that they all have a similar bilene-b chromophore.

Mass spectrometry. The mass spectra of the

pigments (Fig. 10) are very similar, but showed differences in the relative intensities of some ions. The relative intensity of the ion at m/e 371, corresponding to the fragment ion (XVII) formed by loss of both end rings, increases steadily from urobilin 2b, through 2a to 1. In the last it is the base peak whereas the base peak in urobilins 2a and 2b is at m/e 494 corresponding to loss of one end ring. Urobilin 1 showed two major peaks at m/e 208 and 387 not present in the other spectra. Accurate mass determinations were carried out on those ions and that at m/e 371 (Table 2). The ion at m/e 387 in the spectrum of urobilin 1 corresponding to the formula $C_{21}H_{27}N_2O_5$ could be the parent ion of an hydroxydipyrrromethene (XVIII) of molecular weight 388 and the prominent ion at m/e 208 corresponding to the formula $C_{11}H_{14}NO_3$ representing the fragment ion (XIX). The compound XVIII may be present as an impurity in urobilin 1.

Two theoretically possible forms of isomerism in i-urobilin IX α dimethyl ester are (a) prototropic forms (structures generated by consideration of the canonical forms of the chromophore): these are i-urobilin IX α dimethyl ester and iso-i-urobilin IX α dimethyl ester (XX). (b) Stereoisomers: there are two asymmetric carbon atoms in i-urobilin IX α dimethyl ester and therefore four stereoisomeric forms (see Fig. 11).

Protonation of the chromophore of i-urobilin IX α dimethyl ester would certainly be accompanied by charge delocalization and the resulting equivalence of the nitrogen atoms would remove the distinction between i-urobilin IX α dimethyl ester and iso-i-urobilin IX α dimethyl ester. There is no equilibration of urobilins 2a and 2b in acid, and these are therefore most unlikely to be prototropic forms.

The physical properties of diastereoisomers may vary considerably and such forms of i-urobilin IX α dimethyl ester must represent molecules of profoundly different shape. Urobilins 2a and 2b are therefore probably racemic diastereoisomers, since

Table 2. *Accurate masses and formulae of ions*

Formula	m/e (found)	m/e (calc.)
$C_{11}H_{14}NO_3$	208.0981	208.097361
$C_{21}H_{27}N_2O_4$	371.1971	371.197070
$C_{21}H_{27}N_2O_5$	387.1938	387.191984

Fig. 11. Stereoisomeric forms of i-urobilin IX α dimethyl ester.

their physical properties are markedly different although their chemistry is very similar.

Conclusions

The structures proposed for the new pigments are summarized in Table 3.

The sodium amalgam reduction of bilirubin IX α gives a mixture of urobilinogens existing in stereoisomeric modifications and containing pyrrolenone and pyrrolidone end rings, but no compounds containing unsaturated β -substituents have been found. Oxidation of this mixture with methanolic ferric chloride gives a large number of pigments from which verdins, violins and a rhodin have been isolated. Oxidation with aqueous iodine gives principally i-urobilin IX α in stereoisomeric forms and their ring D hydrogenated analogues. The symmetrical and fully unsaturated verdin chromophore appears to exist in one form only, and since the chromophore extends throughout the molecule no ring-hydrogenated forms can exist. Thus verdins can differ only in their β -substituents and from the present pigment mixture the isomeric glaucobilins III α , IX α and XIII α have been isolated. The violins with an asymmetric chromophore and an unconjugated end ring can exist in several forms and pigments of the III α , IX α and XIII α series with both pyrrolenone and pyrrolidone end rings have been isolated. In addition isomeric modifications of the IX α pigments have been found.

Table 3. *Proposed structures of verdins, violins, rhodin and urobilins*

Pigment	Proposed structure (of dimethyl ester)
Verdin 1	Glaucobilin XIII α
Verdin 2	Glaucobilin IX α
Verdin 3	Glaucobilin III α
Violin 1	Mesobiliviolin XIII α
Violin 2a	Isomesobiliviolin IX α
Violin 2b	Mesobiliviolin IX α
Violin 3 (labile)	Mesobiliviolin III α
Violin 3 (stable)	Dihydromesobiliviolin III α or Dihydroisomesobiliviolin IX α
Violin 4	Dihydromesobiliviolin IX α
Violin 5	Dihydromesobiliviolin XIII α
Rhodin	Double bond isomer of corresponding urobilin
Urobilin 1	Not known
Urobilin 2a	Racemic diastereoisomeric forms of i-urobilin IX α + their ring D dehydrogenated derivatives
Urobilin 2b	

A new structure for the rhodin class of pigments has been advanced that is different from that proposed by Siedel (1935).

The origin of the III α and XIII α pigments is obscure, since i-urobilin prepared by oxidation of the urobilinogen mixture is essentially IX α and it is not clear whether III α and XIII α urobilinogens are present or whether perhaps half-molecular

fragments combine under the influence of methanolic ferric chloride to give III α or XIII α violins directly.

Clearly in the preparation of pure bile pigments, especially under alkaline conditions, much care is required to ensure homogeneity of the products.

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