442. *The Chemistry of the Bile Pigments. Part IV.*[1] *Spectrophotometric Titration of the Bile Pigments.*

By C. H. Gray, A. Kulczycka, and D. C. Nicholson.

Spectrophotometric titration curves of four main types of bile pigments and related dipyrryl compounds reveal a predominantly basic character in urobilins, violins, and verdins, the basic strengths decreasing in that order, and a predominantly acidic character in rubins. Implication of these results on the structure of the pigments is discussed.

All naturally occurring bile pigments contain carboxyl groups, separated from the main chromophores by two methylene groups, so that their ionisation is not detectable spectrophotometrically. Thus, the spectra of esters of verdins and of carboxyethylporphyrins are identical with those of the corresponding free pigments.[2,3] Changes in the spectra of bile pigments accompanying change of pH, therefore, arise from addition of protons to, or their removal from, the main chromophores of these compounds.

We have studied rubins, urobilins, verdins, and violins as well as simple dipyrrylmethenes representative of the chromophores of the first two classes of these pigments. We first used McIlwain's buffer solutions,[4] but these are not of constant ionic strength, as shown by an approximately six-fold change of conductivity over their entire range. Their pH range was unsatisfactory for the spectrophotometric study of the urobilins and, less so, of the rubins, which were, therefore, also examined in veronal–acetate buffers which are of constant ionic strength ($\mu = 0{\cdot}1$).[5] These buffers were, however, unsuitable for the investigation of the verdins, but were employed for mesobiliviolin. Results with the two buffer systems were nevertheless almost identical.

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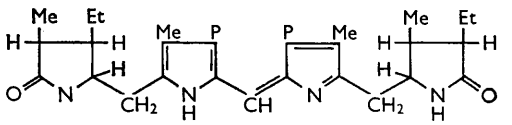

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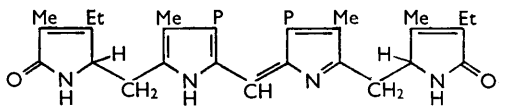

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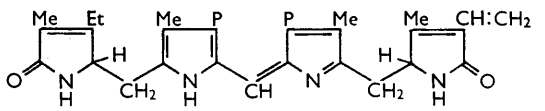

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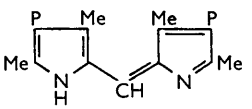


The Urobilins.—The urobilins are orange in aqueous acid solution; in neutral or alkaline solution they are yellow. Stercobilin (I), i-urobilin (II), d-urobilin (III *), and the dipyrrylmethene (IV) gave similar spectroscopic absorption curves, all of which varied

* For an alternative formula see ref. 6.

[1] Part III, preceding paper.
[2] Lemberg and Legge, "Haematin Compounds and Bile Pigments," Interscience Publ. Inc., New York, 1949, p. 75.
[3] Holt and Jacobs, *Ann. J. Bot.*, 1954, **41**, 710.
[4] "Handbook of Chemistry and Physics," Chemical Rubber Publ. Co., 1950, p. 1487.
[5] Neuberger and Scott, *Proc. Roy. Soc.*, 1952, *A*, **213**, 307.
[6] Siedel and Meier, *Z. physiol. Chem.*, 1936, **242**, 101.

Spectrophotometric titration data for the urobilins in veronal–acetate buffers.

Pigment	Maximum in acid (mμ)	pH	Maximum in alkali (mμ)	pH	p*K*	Posn. (mμ) of isosbestic point
Dipyrrylmethene (IV) ...	480	6·80	455—460	9·75	8·50	450
Stercobilin (I)	490	6·15	452·5—455	8·70	7·60	458
i-Urobilin (II)	491	5·90	455—460	8·60	7·40	460
d-Urobilin (III)	491—492	5·75	460	8·40	7·20	460

in the same way with pH. The spectral absorption curves of stercobilin shown in Fig. 1 are typical.

Differences in the spectroscopic behaviour between the free forms and the hydrochlorides of urobilins and allied dipyrrylmethenes have been reported by Pruckner and

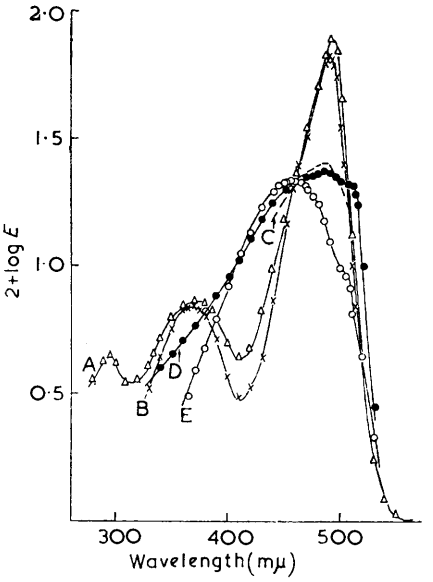


FIG. 1. *Spectrophotometric absorption curves of stercobilin* (I) *in veronal–acetate buffers*: A, pH 1·0 (*without buffer*); B, pH 7·0; C, pH 8·4; D, pH 9·1; E, pH 12·0 (*without buffer*).

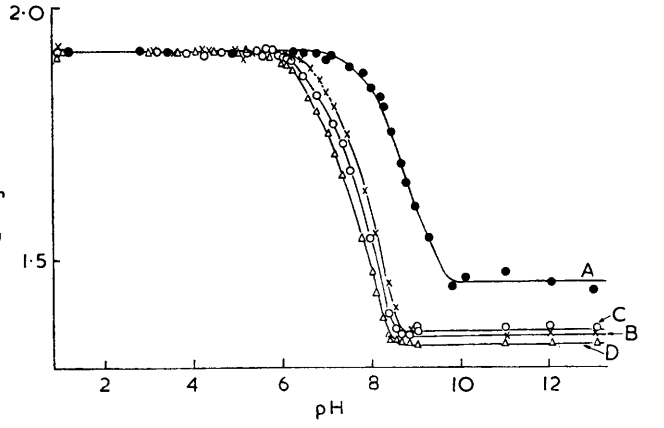


FIG. 2. *Spectrophotometric titration curves of urobilins in veronal–acetate buffers*: A, *dipyrrylmethene* (IV); B, *stercobilin* (I); C, *i-urobilin* (II); D, *d-urobilin* (III). *Low and intermediate* pH *plots refer to an absorption maximum at* ca. 490 mμ; *high* pH *plots refer to an absorption maximum at* ca. 460 mμ.

Stern.[7] The free compounds in dioxan gave absorption curves resembling those found by us for solutions of high pH; hydrochlorides in the same solvent gave curves resembling those shown here for solutions of low pH. The spectrophotometric titration curves and the corresponding data for the pigments in the veronal–acetate buffers are shown in Fig. 2 and in the Table respectively. The absorption curves at pH above 9 had additional

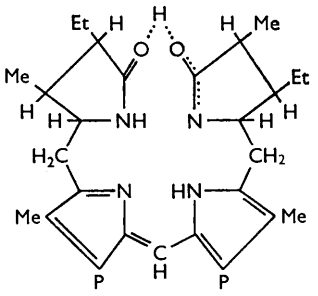
[7] Pruckner and Stern, *Z. phys. Chem.*, 1937, *A*, **180**, 25; 1938, *A*, **182**, 117.

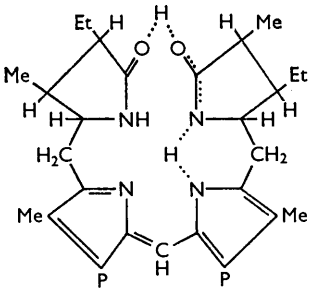
maxima at about 510 mμ; they did not then pass through the isosbestic point owing to complex formation, especially with i-urobilin at pH 12—13. The titration curves in McIlwains buffers can only be determined with accuracy on the acid side of the pK, which was only about 0·1 of a pH unit higher than that in the veronal–acetate buffer. The isosbestic points and the molecular extinction coefficient in acid and alkaline solution were also the same for the two buffers.

The dipyrrylmethene group present in the urobilins contains one nucleophilic pyrroline-nitrogen atom; these compounds, therefore, have a predominantly basic chromophore. The titration curves of the urobilins show that these pigments may be arranged in the decreasing order of their basic strength: stercobilin, i-urobilin, and d-urobilin, the conjugate acids having pK 7·60, 7·40, and 7·20 respectively. The stronger basic character of stercobilin must be attributed to the saturated nature of the terminal rings. In stercobilin the electron density about the pyrroline-nitrogen atom of the dipyrrylmethene chromophore, being undisturbed by effects from remote parts of the molecule, may be assumed more suitable for external co-ordination (*e.g.*, of a proton) than it is around the nitrogen in the other molecules.

Recently, Lemberg has suggested a monolactam structure for stercobilin, with a hydrogen bonding between the two terminal oxygen atoms (V). Such a structure, unlike the bislactam form, provides two replaceable N-hydrogen atoms and two electron-donating nitrogen atoms, thus accounting for the inclusion of one metal atom in each molecule of metal complex.[8] In one end ring of stercobilin there is thus a nucleophilic nitrogen atom which might co-ordinate to itself the proton formulated as part of the dipyrrylmethene unit. A further hydrogen-bonded structure would arise as shown in (VI).

(V)





(VI)

Stercobilin has no unsaturation in the end ring to restrain by mesomeric effects the electrons presumed to take part in such hydrogen bonding which would, therefore, be strong. The electrons of the dipyrrylmethene group thus partially relieved of their rôle in binding the hydrogen atom of the pyrrole group, will have a greater ability to co-ordinate an extramolecular proton. In i-urobilin, the end rings are unsaturated and the electrons of their nitrogen atoms being, in consequence, less localised than the corresponding electrons in stercobilin, will less readily engage in hydrogen-bond formation. The electrons of the dipyrrylmethene chromophore, having, therefore, to bind the hydrogen atom on the pyrrole nitrogen more completely than in stercobilin, will be less readily available for co-ordination of an external proton. In d-urobilin, the presence of an additional centre of unsaturation in, or attached to, one end ring, enhances the effect described for i-urobilin; the dipyrrylmethene group of this pigment should, therefore, even less readily co-ordinate to itself an external proton. Thus, the decrease in nucleophilic properties of the urobilins, in order stercobilin, i-urobilin, and d-urobilin, is understood.

The dipyrrylmethene (IV), the conjugate acid of which dissociates with pK 8·50, is

[8] Lemberg, personal communication.

an even stronger base than stercobilin; the greater availability of electrons in this compound may be readily explained by a positive inductive or hyperconjugative effect from the α-methyl groups.

Confirmation that the pyrroline-nitrogen atom is the spectroscopically active nucleophilic centre of the urobilins was sought by the examination of stercobilinogen, the dihydro-derivative of stercobilin, which contains no pyrroline-nitrogen atom. The compound, however, was too labile for determination of its pure spectrum, being rapidly autoxidised to stercobilin. The dipyrrylmethane obtained by reduction of compound (IV) with sodium amalgam was also unstable and could not be studied in buffer solutions owing to powerful absorption of the latter in the ultraviolet region. It was, nevertheless,

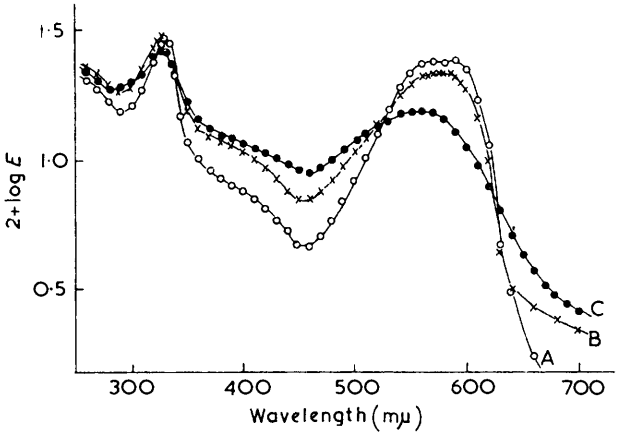


FIG. 3. *Spectrophotometric absorption curves for mesobiliviolin* (VII) *in McIlwain buffers*: A, pH 1 (*without buffer*); B, pH 2·2; C, pH 4·4.

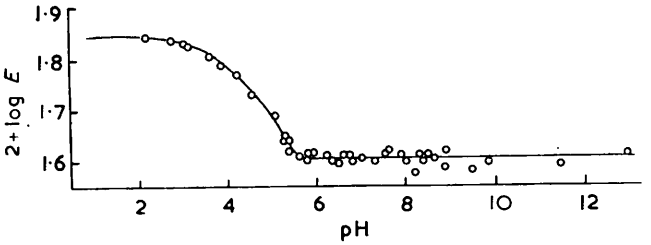
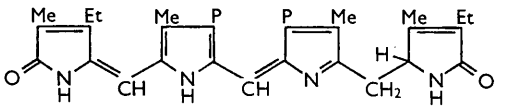


FIG. 4. *Spectrophotometric titration curve for the* 550—585 mμ *absorption maximum of mesobiliviolin* (VII) *in veronal–acetate buffer.*

clearly established that this substance, which also contains no pyrroline-nitrogen atom, in absence of buffer absorbs more strongly, at its maximum (268 mμ) in alkaline than in acid solution and, therefore, resembles a rubin (see below).

Violins.—Aqueous solutions of mesobiliviolin are red-violet at pH values above 5 and blue-violet or blue at pH values below 4. Absorption curves for mesobiliviolin (VII) in McIlwain's buffers are shown in Fig. 3. As with urobilins, but less readily so, alkaline solutions of mesobiliviolin rapidly form metal complexes, the solutions in consequence developing red fluorescence.

Fig. 4 shows the spectrophotometric titration curve obtained for the 550—585 mμ absorption maximum of mesobiliviolin in veronal–acetate buffer; points below pH 3 were



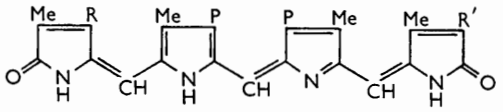
(VII)

obtained in hydrochloric acid, the solution then being of different ionic strength. In veronal–acetate buffer full titration curves were obtained for only a few pH values and these were identical with those obtained for solutions in McIlwain buffers (Fig. 3).

As with urobilins and consistent with the presence of a pyrroline-nitrogen atom, mesobiliviolin shows an overall decrease of maximal optical absorption with increase of pH; in strongly acid solution, absorption is particularly enhanced. The chromophore of this pigment is therefore basic, the conjugate acid dissociating with pK about 4·0. Free mesobiliviolin is, then, a weaker base than any of the urobilins. This is expected, since in this pigment electrons of the nucleophilic nitrogen atom are in a more extensive chromophore (three conjugated rings instead of two, as in urobilins), and are, therefore, less readily available for external co-ordination of a proton. The chromophore also differs from that of the urobilins in that it contains an electronegative carbonyl group. The effect of this is considered below.

Verdins.—Aqueous solutions of biliverdin and dihydrobiliverdin are green-blue and those of glaucobilin are blue. The solutions are almost the same colour whether acidic or alkaline. Spectroscopic data and absorption curves for the verdins are given in the preceding paper.

Of the verdinoid pigments, glaucobilin (VIIIa), dihydrobiliverdin (VIIIb), and biliverdin (VIIIc) were examined. These compounds in McIlwain's buffers show inflections

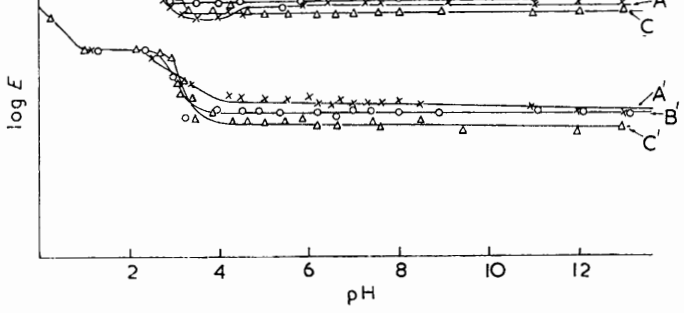


(VIIIa) R = R′ = Et (VIIIb) R = CH:CH$_2$; R′ = Et

(VIIIc) R = R′ = CH:CH$_2$ or R = Et; R′ = CH:CH$_2$

of their spectrophotometric titration curves at pH values below 4 (Fig. 5); no attempt was, therefore, made to reproduce these curves in veronal–acetate buffers. The verdins, especially glaucobilin, were unstable at pH between 2·5 and 5, absorption at both maxima

FIG. 5. *Spectrophotometric titration curves of verdins in McIlwain buffers*: A, *biliverdin* (VIIIc) (~360 mμ max.); B, *dihydrobiliverdin* (VIIIb) (~360 mμ max.); C, *mesobiliverdin* (VIIIa) (~360 mμ max.); A′, *biliverdin* (~660 mμ max.); B′, *dihydrobilirubin* (~660 mμ max.); C′, *mesobiliverdin* (~660 mμ max.). *Positions of curves have been adjusted for convenience of illustration; no scale for the ordinate is therefore given.*



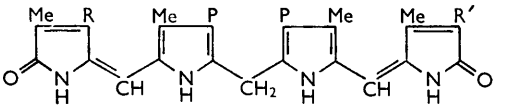
rapidly decreasing. The maximum at 660 mμ was replaced by a new one at 540—580 mμ, and increased absorption between 350 and 250 mμ accompanied the disappearance of the maximum at 365 mμ. These changes, in the case of glaucobilin and especially at pH 3·4, are accompanied by development of a violet colour and indicate conversion into a purpurin. Because of this instability, titration curves for verdins derived from complete absorption curves show a minimum between pH 2·5 and 5. More accurate curves for these pigments

were, therefore, obtained by determining the optical density first at the lower, less stable, maximum within one minute of preparation of the solutions. Curves so obtained are given in Fig. 5.

Verdins, like violins and urobilins, provide titration curves showing greater maximal absorption at low pH than at high pH. Instability of the verdins at low pH precludes the derivation of an exact pK value for the conjugate acids of these pigments, an approximate value being 3. Nevertheless, titration curves for the verdins are without inflections over the range of pH where these are found for violins and urobilins. The verdins are, therefore, much weaker bases than violins or urobilins and this is expected, since the electrons of their pyrroline-nitrogen atoms, being included in the most extensive resonating system found in the bile pigments, are the least readily available for the co-ordination of an external proton. For the same reason, the pK of biliverdin is lower than that of dihydrobiliverdin or glaucobilin.

Both verdins and violins contain electronegative carbonyl groups in their chromophores; these must also be partly responsible for the lower basic strength of these pigments than of the urobilins. Complexity of the chromophores of the basic bile pigments increases from urobilins (two conjugated pyrrole rings), through violins (three conjugated rings with one carbonyl group), to verdins (four conjugated rings with two carbonyl groups). Attachment of protons to these chromophores would be expected to disturb the electronic structures of the urobilins most, of the violins moderately, and of the verdins least. It is significant that the ratios of optical absorption of the protonated molecule to that of the unprotonated molecule decrease in this order.

Rubins.—Aqueous solutions of rubins are yellow, whether they are acid or alkaline, having one broad maximum in the blue, the wavelength of which decreases in the order bilirubin (IXa), dihydrobilirubin (IXb), dimesobilirubin (IXc).[1] Absorption spectra of



(IXa) R = R′ = CH:CH$_2$

(IXb) R = CH:CH$_2$, R′ = Et or *vice-versa*

(IXc) R = R′ = Et

these compounds are similar; those for mesobilirubin at various pH values in McIlwain's buffers are typical and are presented in Fig. 6, because this pigment contains the same β-substituents as i-urobilin, mesobiliviolin, and glaucobilin. The titration curves for the rubins and the analogous dipyrrylmethenes (X), (XI) are shown in Fig. 7, for solutions in McIlwain's buffers. Because of the instability of rubins toward light and air, these curves were constructed by measuring absorption rapidly, first at the maximum and immediately afterwards in the region of the isosbestic point, always within 3 min. of preparation of solution. In contrast to these results, Williams and Ruz, and Martin,[9] found a decrease of optical density with increasing pH for solutions of bilirubin but describe no precautions against the instability of their stock solution of pigment. At pH's below 5, the solutions sometimes developed turbidity, it then being difficult to obtain reproducible optical densities. Absorption curves of these solutions, especially those of bilirubin (the least soluble), did not always pass through the isosbestic point. For bilirubin, an anomalous minimum, which disappeared with time, appeared in the titration curve.

Rubins form no metal complexes;[10] they therefore cannot contain nucleophilic nitrogen

[9] Williams and Ruz, *Rev. Soc. Argentina de Biol.*, 1943, **19**, 401; Martin, *J. Amer. Chem. Soc.*, 1949, **41**, 1230.

[10] Ref. 2, pp. 108, 123.

atoms. They thus lack the basic character of urobilins, violins, and verdins; and, of all the bile pigments, they alone do not form hydrochlorides. The classical structure for bilirubin with hydroxyl groups in the end α-positions contains one formally nucleophilic nitrogen atom in each end ring; the bislactam form (IXa) contains no nucleophilic nitrogen

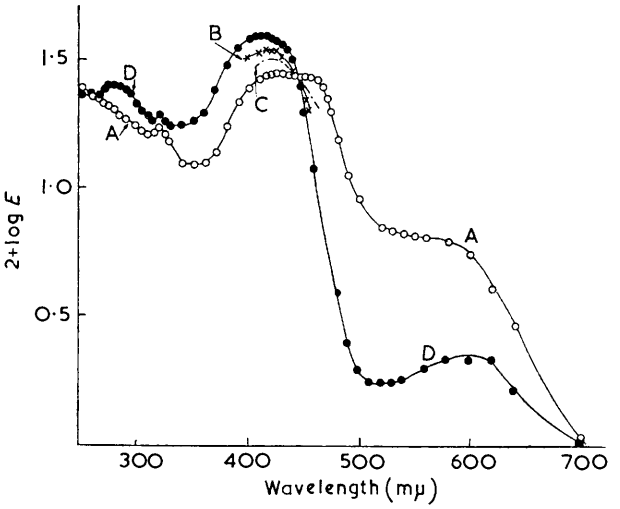
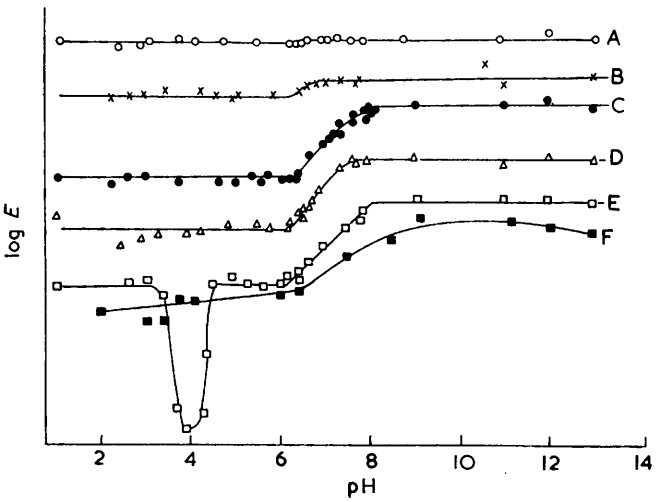


FIG. 6. *Spectrophotometric absorption curves of mesobilirubin* (IXc) *in McIlwain's buffers*: A, pH 1 (*without buffer*); B, pH 7; C, pH 7·8; D, pH 13 (*without buffer*).

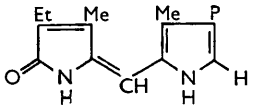
FIG. 7. *Spectrophotometric titration curves of rubins and allied dipyrrylmethenes in McIlwain buffers*: A, *the dipyrrylmethene* (X); B, *the dipyrrylmethene* (XI); C, *mesobilirubin* (IXc); D, *dihydrobilirubin* (IXb); E, *bilirubin* (IXa); F, *readings obtained for bilirubin solution after* 1 *hr. Positions of curves have been adjusted for convenience of illustration; no scale for the ordinate is therefore given.*

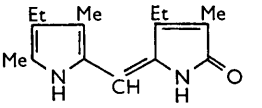


atoms and is, therefore, more compatible with the above facts. The dipyrrylmethene units in bilirubin each contain a carbonyl group capable of accommodating the anionic charge resulting from the dissociation of an NH group, and the rubins should, therefore, be well stabilised in the anionic form. All four *N*-hydrogen atoms in the bislactam structure should, therefore, individually be more easily ionised than the corresponding acidic hydrogen atom in pyrrole itself. A predominantly acidic character for these pigments is also consistent with the prototropy occurring in bilirubin as described in the preceding paper, because these two properties often co-exist.[11] The increased absorption of solutions

[11] Ingold, " Structure and Mechanism in Organic Chemistry," G. Bell, London, 1953, pp. 543—550.

of high pH, therefore, probably corresponds to the separation of a proton from a molecule of the free rubin. The titration curves show that bilirubin, dihydrobilirubin, and mesobilirubin have pK about 7·1, 7·2, and 7·3, respectively. Because, however, of the instability of the rubins, their irregular behaviour below pH 5, and the small increments of optical density with pH, these values are not accurate. Nevertheless, both in McIlwain's buffer and veronal–acetate buffer, the same pattern of absorption was always observed. The apparent increase of acid strength in the order mesobilirubin, dihydrobilirubin, bilirubin is consistent with the same order for increasing unsaturation and tolerance of anionic change.

(X) 

 (XI)

Isoneoxanthobilirubic acid (X) and 3,4'-diethyl-5-hydroxy-4,3',5'-trimethyldipyrryl methene (XI) resemble the hydroxydipyrrylmethene units of rubins. Both give yellow aqueous solutions, the colour being the same in acid and alkali; the titration curves are given in Fig. 7. Isoneoxanthobilirubic acid in all aqueous solutions has an absorption maximum at 395—400 mμ, the optical density being almost independent of pH (Fig. 7). The dipyrrylmethene (XI) had an absorption maximum at 395—410 mμ in acid buffers and at 375—397 mμ in alkaline buffers, with one isosbestic point at 420 mμ. There is little indication of acidity in these compounds; like the rubins, however, they do not form metal complexes or hydrochlorides and cannot contain electron-donating nitrogen atoms. They are accordingly better represented by the lactam formulæ shown than as hydroxypyrroles.[12]

Experimental

Stercobilin and d-Urobilin.—Stercobilin hydrochloride, $[\alpha]_D$ −3700° in $CHCl_3$, and d-urobilin hydrochloride, $[\alpha]_D$ +5000 in $CHCl_3$, were isolated as previously described [13] and were recrystallised from chloroform. $10^{-3}\varepsilon$ = 92·9 at 496 mμ for stercobilin in $CHCl_3$, and 93·7 at 499 mμ for d-urobilin in $CHCl_3$.

i-Urobilin.—i-Urobilin was prepared from bilirubin by reduction to mesobilirubinogen with sodium amalgam, followed by oxidation in glacial acetic acid.[6] Final recrystallisation of the hydrochloride from chloroform containing a little dry acetone afforded a product with $\lambda_{max.}$ (in $CHCl_3$) 499 mμ (ε 7·21 × 10^{-4}).

Mesobiliviolin.—Mesobilirubinogen, prepared as above from bilirubin (100 mg.), was dissolved in methanol (10 ml.). 20% Ferric chloride in concentrated hydrochloric acid (1 ml.) was added and the solution was boiled under reflux for 15 min. After addition of water (30 ml.), pigments were re-extracted into ether (final volume, 300 ml.). Urobilin was removed by washing the ethereal extract with water until no further yellow colour appeared in the washings. The washed ethereal solution was exhaustively extracted with 2·8N-hydrochloric acid, and violet pigment was then extracted from the acid extract into chloroform (final volume, 80 c.c.). The chloroform solution was washed free from acid, filtered through chloroform-impregnated paper, and evaporated to dryness. The dry residue of violinoid pigment, dissolved in the smallest possible quantity of 2 : 1 chloroform–ether, was brought on to a column of magnesium oxide (35 × 1 cm.). Elution with the same solvent caused separation of the pigment into an immobile fraction of indefinite purple colour and a mobile band. Further development resolved the mobile band into (*a*) a small upper verdinoid band, (*b*) an intermediate violet band (main fraction), and (*c*) a lower red band (smallest). Band (*b*) was composed of pure violin which had $10^{-3}\varepsilon$ = 20·0 at 565 mμ and 21·6 at 327·5 mμ in 5% w/v HCl–MeOH, 12·0 at 565 mμ and 18·5 at 327 mμ in $CHCl_3$, and 23·8 at 602·5 mμ and 22·3 at 327·5 mμ in $CHCl_3$ shaken with 2·8N-HCl. The zinc complex in ethanol was blue with bright red fluorescence and had $\lambda_{max.}$ at 632·5, 582·5, and 340 mμ (lit.,[14] 630·6, 580—566 mμ in EtOH).

[12] Fischer and Orth, " Die Chemie des Pyrrols," Academische Verlagsges., Leipzig, 1937, Vol. II, p. 649.
[13] Gray and Nicholson, *J.*, 1958, 3085.
[14] Siedel, *Z. physiol. Chem.*, 1935, **237**, 8.

In this preparation, a larger quantity of ferric chloride, prolonged heating, or too long contact with air, results in formation of purpurinoid pigments, the visible absorption maxima of which are shifted by about 10 mμ towards lower wavelength. These are readily distinguished from true violin by their failure to undergo oxidation to verdin on further treatment with ferric chloride.

Dihydrobilirubin and Mesobilirubin.—These were obtained by quantitative hydrogenation of bilirubin at atmospheric pressure.

Biliverdin, Dihydrobiliverdin, and Mesobiliverdin (*Glaucobilin*).—These were prepared by oxidation of bilirubin, dihydrobilirubin, and mesobilirubin (see below), respectively, with ferric chloride. Methods used for the production of these pigments and their spectroscopic characteristics are described in the preceding paper.

Glaucobilin.—Glaucobilin was also obtained by oxidation of mesobilirubinogen. The mesobilirubinogen prepared by reduction of bilirubin (200 mg.) with 2·5% sodium amalgam (2 × 10 g.), as mentioned in the description of i-urobilin, was boiled under reflux in methanol (10 ml.) and 20% ferric chloride solution (1 ml.) for 45 min. Pigments were brought into chloroform solution by the procedure described for mesobiliviolin, and the washed solution was dried by filtration. Initial chromatography on magnesium oxide separated an immobile band (*a*) and a mobile band (*b*). Elution with 2 : 1 chloroform–ether caused slight development of band (*a*) into 5 fractions (violet, yellow, violet, green-yellow, and pink) of increasing mobility. Band (*b*) separated into a red-violet (minor and more mobile) and a blue fraction (major and less mobile). The pigment of the blue band in chloroform had maxima at 635—640 and 370 mμ and was united with the verdinoid band obtained in the preparation of mesobiliviolin. These combined fractions (3·87 mg.) were rechromatographed and found to be homogeneous. The spectral characteristics are given in the preceding paper.

3,4′-*Diethyl*-5-*hydroxy*-4,3′,5′-*trimethyldipyrrylmethene* (XI).—Ethyl hydroxyiminoacetate was prepared and purified by Corwin and Ellingstone's method.[15] It did not crystallise. Distillation afforded a slightly yellow, odourless product, b. p. 151—152°/24 mm., in 47% yield.

Ethyl 4-acetyl-3,5-dimethylpyrrole-2-carboxylate, m. p. 148°, was prepared by the Knorr condensation of the oxime-ester and acetylacetone in 55% yield (lit.,[16] m. p. 143°, yield, crude, 55%).

Ethyl 4-ethyl-3,5-dimethylpyrrole-2-carboxylate, m. p. 94°, was prepared [17] from the acetyl derivative in quantitative yield by hydrogenation at high temperature and pressure.

3-*Ethyl*-2,4-*dimethylpyrrole* (*cryptopyrrole*).—The last-mentioned ester (33 g.) and potassium hydroxide (20 g.) in water (100 ml.) were boiled for 3 days under nitrogen. The solution was saturated with sodium chloride and extracted with ether (10 times). The extracts were dried (K_2CO_3), the solvent was removed, and the residue distilled, to give the pyrrole, b. p. 90°/14 mm. (18 g., 82%). Fischer and Orth [18] obtained the pyrrole in an overall yield of 62% by one-step reduction of the 3-acetyl group and decarboxylation with hydrazine hydrate in alcoholic sodium ethoxide. The recorded method [19] of bromination gave 5-bromo-3,4′-diethyl-4,3′,5′-trimethyldipyrrylmethene hydrobromide, m. p. 148°, and thence by potassium acetate in boiling acetic acid,[20] 3,4-diethyl-5-hydroxy-4,3′,5′-trimethyldipyrrylmethene, m. p. 245—248°.

The corresponding methane was obtained by reduction of this compound in methanol with sodium amalgam.

Diethyl 3,3′,5,5′-*Tetramethyldipyrrylmethene*-4,4′-*dicarboxylate* (IV).—Diethyl hydroxyiminomalonate, prepared by Cherchez's method,[21] had b. p. 175—185°/15 mm. (yield 75%) (lit.,: yield 80—90%, b. p. 172°/12 mm.).

Thence the method of Gresham *et al.*[22] gave 4-acetyl-5-oxohexanoic acid, b. p. 150—170°/1·5 mm. (36%) (lit.: 124—142°/1 mm., 42%), m. p. 76—80° (from hexane; 15%).

To a stirred solution of this acid (86 g., 1·5 mol.) in glacial acetic acid (260 ml.) containing anhydrous sodium acetate (130 g.) and zinc dust (111 g.), was added diethyl hydroxyiminomalonate (94·7 g., 1·47 mol.) in acetic acid (120 ml.) and water (50 ml.) at a rate to maintain

[15] Corwin and Ellingstone, *J. Amer. Chem. Soc.*, 1944, **66**, 1150.
[16] Ref. 12, Vol. I, p. 193.
[17] Eisner, Lichtarowicz, and Linstead, *J.*, 1957, 733.
[18] Ref. 12, Vol. I, p. 54.
[19] Ref. 12, Vol. I, p. 73.
[20] Ref. 12, Vol. II, p. 114.
[21] Cherchez, *Bull. Soc. chim. France*, 1930, **47**, 1279.
[22] Gresham, Jansen, Shaver, and Bears, *J. Amer. Chem. Soc.*, 1951, **73**, 2345.

refluxing. Then the mixture was boiled for a further 2 hr. and poured on ice (2 kg.). After storage in a refrigerator overnight, the crystalline 4-ethyl hydrogen 2,5-dimethylpyrrole-2,4-dicarboxylate was filtered off and dried. It (80 g., 67%) had m. p. 158—160°, unchanged by recrystallisation (lit.:[23] 59%, m. p. indefinite; after several crystallisations from benzene, m. p. 154—156°).

This product gave the orange hydrochloride, m. p. 238°, of ester (IV) in 70% yield by Fischer and Morgenroth's method.[24]

The corresponding dipyrrylmethane was obtained by reduction of the methene in methanol with sodium amalgam.

Isoneoxanthobilirubic Acid (X).—A mixture of neoxanthobilirubic and isoneoxanthobilirubic acid obtained from mesobilirubin (2·07 g.) by fusion with resorcinol (60 g.), according to the method of Fischer and Hess,[25] was esterified by hydrogen chloride in methanol. Fractional crystallisation from ethanol–methanol afforded the iso-ester (190 mg.), m. p. 200° (lit.,[26] 203°). The free acid (X) (15 mg.), obtained by hydrolysis of the ester, crystallised from methanol–acetic acid, had m. p. 232° (lit., 240—242°).

Determination of Spectrophotometric Titration Curves.—A Hilger H 700 spectrophotometer with a quartz prism was used. Wavelength setting and optical density readings were checked with calibrated didymium glass and rhodium quartz filters respectively. Quartz 1 cm. cells were used for all compounds except the rubins; for these the 4 cm. cells were used.

McIlwain buffer solutions [4] of various pH values were prepared immediately before spectrophotometry from " AnalaR " chemicals in distilled carbon dioxide-free water which had passed through an Algastat Minor Type C 403 portable deioniser. They were stored in bottles coated internally with paraffin wax. Methanolic solutions of the compounds investigated were prepared in such concentrations that the addition of 0·5 ml. to 4 ml. of buffer solutions gave a solution with the optical density between 0·7 and 0·8 at the spectroscopic maxima. The same pipettes being used throughout, these volumes were transferred to 6″ stoppered tubes, shaken, and immediately examined. After spectroscopy, the pH of each solution was determined on an E.I.L. 23A pH-meter, standardised as described in Part II of this series. Similar solutions prepared with 0·1N-hydrochloric acid, 0·1N-, 0·01N-, and 0·001N-sodium hydroxide were also examined, but the pH's of these were not confirmed potentiometrically. Solutions of urobilins prepared in this way were sufficiently stable to permit determination of complete spectral absorption curves over a wide range of pH (Fig. 2). With other pigments, particularly the verdins, solutions of pH 2—5 developed turbidity and then had lower optical density at the maxima. In such cases, complete spectral absorption curves were determined only for a few solutions, so as to establish the isosbestic points; at other pH values absorption at the maxima were rapidly determined, together with some at wavelengths close to and including the isosbestic point. Titration curves for the urobilins were also determined in this way, since prolonged contact with glass, and thus metal-complex formation at high pH, was avoided.

Veronal–acetate buffers of constant ionic strength (μ 0·1) were prepared from 0·18N-sodium chloride (" AnalaR ") and 0·12N-hydrochloric acid (" AnalaR ") and 0·06M-sodium acetate–sodium barbitone, the necessary volumes being calculated from the formulæ derived by Neuberger and Scott [5] and the Henderson–Hasselbach equation. Titration curves were determined with these buffers in the way described for the McIlwain buffers. These buffers were stored in Polythene bottles. With solutions so prepared, metal-complex formation by urobilins in alkaline solution did not occur.

Spectrophotometric titration curves were determined for different concentrations of stercobilin, d-urobilin, glaucobilin, biliverdin, the rubins, and the dipyrrylmethenes. No variation with concentration of pigment was observed.

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[23] Kleinspehn, *J. Amer. Chem. Soc.*, 1955, **77**, 1547.
[24] Fischer and Morgenroth, *Annalen*, 1928, **466**, 165.
[25] Fischer and Hess, *Z. physiol. Chem.*, 1931, **194**, 209.