627. *The Chemistry of the Bile Pigments. The Structures of Stercobilin and d-Urobilin.*

By C. H. Gray and D. C. Nicholson.

Stercobilin and d-urobilin have been degraded with the formation of fragments from the end ring and middle ring of each. The structure of stercobilin as a hydrogenated i-urobilin, *i.e.*, mesobilene-b, has been confirmed and the β-positions of the end rings have been established as the sites of attachment of the extra hydrogen atoms. The production of ethylmethylmaleimide and hæmatinimide by the oxidation of d-urobilin and the quantitative hydrogenation of this pigment suggest that it is related to i-urobilin in that one ethyl group is replaced by a vinyl group. The isolation of two new urobilinoid pigments, namely, d-urobilin-IXα and racemic dehydro-i-urobilin, is described.

Most bile pigments are tetrapyrrolic compounds derived from biliverdin [4 : 5-di-2′-carboxyethyl-1 : 3 : 6 : 7-tetramethyl-2 : 8-divinylbilatriene-a,b,c (I)] by different degrees of reduction. They are classified according to the number and position of the double bonds joining the bridge carbon atoms (a,b,c, in I) to the pyrrole nuclei.[1] Biliverdin is formally derived from protoporphyrin-IX (II) by oxidative removal of the α-carbon linkage, and all naturally occurring bile pigments are therefore assumed to be IXα-compounds in that the arrangement of the β-substituents corresponds to that in biliverdin. Biliverdin and a number of related compounds have been synthesised and their IXα-structures established beyond doubt. Analytical evidence of this has also been obtained.[2] Pigments in which the vinyl groups have been reduced to ethyl groups are given the prefix " meso " because of their formal relation to mesoporphyrin (III). The structure usually assigned to biliverdin is the lactim form of the bislactam (I), but the lactam structure is more consistent with recent knowledge of the " hydroxypyrroles " [3] and of the bile pigments,[4] and is therefore used in this paper.

An important group of bile pigments includes the urobilins which are thought to be mesobilenes-b. These include stercobilin [5] ($[\alpha]_D$ −4000° in $CHCl_3$), d-urobilin [5,6] ($[\alpha]_D$ +5000° in $CHCl_3$), and the optically inactive i-urobilin. i-Urobilin receives the trivial name, urobilin-IXα, although all the naturally occurring bile pigments have the IXα-arrangement of β-side chains. Of these compounds only the structure of i-urobilin has been established by degradation and synthesis [7] as 4 : 5-di-2-carboxyethyl-2 : 8-diethyl-1 : 3 : 6 : 7-tetramethylbilene-b (XIV or XVI).

Elementary analyses suggest that stercobilin [8] and d-urobilin [6] are tetrahydro- and dehydro-derivatives, respectively, of i-urobilin. The two pigments cannot, therefore, be structural isomers or enantiomorphs as suggested by With [9] although the racemisation of d-urobilin described in this paper implies the existence of a true enantiomorph of that pigment. Both pigments presumably contain a dipyrromethene structure since their absorption spectra closely resemble those of i-urobilin [6] and the simple dipyrrylmethenes.[10] By the action of concentrated sulphuric acid stercobilin is converted into a glaucobilin (*i.e.*, a mesobiliverdin) which is identical with synthetic glaucobilin IXα; [8] stercobilin

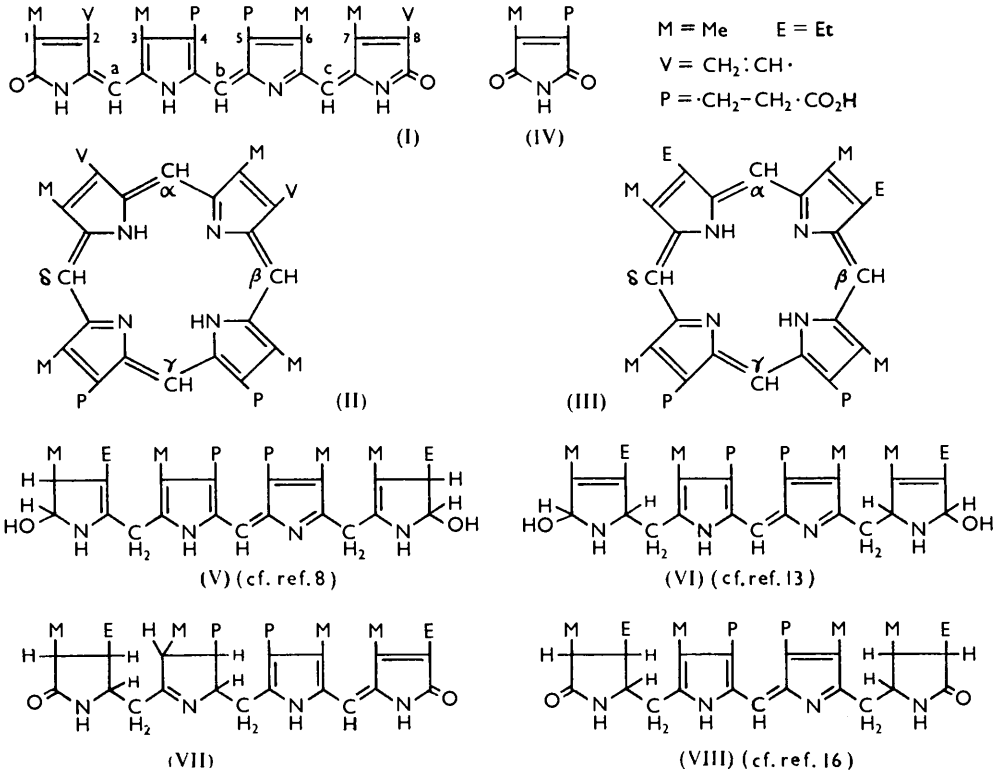
[1] Lemberg and Legge, " Haematin Compounds and the Bile Pigments," Interscience Publ., Inc. New York, 1948, p. 107.
[2] Gray, Nicholson, and Nicolaus, *Nature*, 1958, **181**, 183.
[3] Decker and Plieninger, *Annalen*, 1956, **598**, 198.
[4] Birch, Smith, and Smith, personal communication.
[5] Lowry, Ziegler, and Watson, 1952, " Recent Studies of the Urobilin Problem," *Bull. Univ. Minnesota Hosp. & Med. Foundn.*, No. 7, 1952.
[6] Lowry, Cardinal, Collins, and Watson, *J. Biol. Chem.*, 1956, **218**, 633, 641.
[7] Siedel and Meier, *Z. physiol. Chem.*, 1936, **242**, 101.
[8] Fischer and Halbach, *ibid.*, 1935, **238**, 59.
[9] With, " Biology of the Bile Pigments," Arne Frost-Hansen, Copenhagen, 1954, p. 17.
[10] Pruckner and Stern, *Z. phys. Chem.*, 1938, *A*, **182**, 117.

must therefore be a IXα-isomer and the same must be true of d-urobilin which undergoes smooth reduction to mesobilirubinogen (XIII) or (XV), the mesobilane corresponding to i-urobilin. The absence of ethylmethylmaleimide as an oxidation product of stercobilin has been noticed [8] and this has been interpreted as indicating that the extra hydrogen atoms in this pigment are located in the end rings. However, no further direct evidence has been put forward concerning the structures of stercobilin or d-urobilin.

Stercobilin

Oxidative Products of Stercobilin.—The stercobilin used in our experiments was prepared by Watson's method,[11] by extraction from the fæces of normal subjects or patients with hæmolytic disease, and purified by repeated recrystallisation of the hydrochloride.

Preliminary experiments showed that stercobilin hydrochloride is fairly slowly oxidised by 0·74N-chromium trioxide solution in sulphuric acid, the pigment utilising about 38 equivalents per molecule and about 5 atoms of carbon per molecule being oxidised to carbon dioxide. Chromatographic examination of the products revealed the presence of acetic



acid, succinic acid, hæmatinimide (IV), and ethylmethylsuccinimide. Similar oxidations of larger quantities of the hydrochloride resulted in the conversion of about 37% of the nitrogen to ammonia or a volatile amine and afforded sufficient succinic acid and ethylmethylsuccinimide for comparison with authentic compounds. The neutral fraction, invariably obtained as a weakly dextrorotatory oil, had ultraviolet and infrared spectra similar to those of authentic DL-ethylmethylsuccinimide, identity being confirmed by elementary analysis and hydrolysis to the acid. The natural imide was never obtained

[11] Watson, *J. Biol. Chem.*, 1934, **105**, 469.

crystalline and appeared to contain traces of unidentified substances which caused slight differences in its ultraviolet spectrum compared with those of authentic imide and the product of dry distillation of ammonium *meso*-ethylmethylsuccinate. Authentic DL-ethylmethylsuccinimide, prepared and isolated by a method similar to that used by Linstead and Whalley [12] for DL-αα′-dimethylsuccinimide, proved to be a readily crystallisable compound of low melting point and it is possible that the natural product was a mixture of the *meso*- and DL-isomers. The stereochemistry of these isomers and hence of stercobilin will form the subject of a subsequent paper.

Ethylmethylmaleimide and dihydrohæmatinimide appear to be absent from the oxidation products of stercobilin. Hæmatinimide, although shown chromatographically to be a product of oxidation from stercobilin, was never obtained crystalline. However, stercobilinogen, the dihydro-derivative of stercobilin obtained in a non-crystalline form by amalgam or catalytic reduction, afforded hæmatinimide in about 50% of the yield expected if stercobilin is assumed to contain two 3-carboxyethyl-4-methylpyrrole rings per molecule.

The Structure of Stercobilin.—Oxidative degradation of stercobilin to ethylmethylsuccinimide shows that the end rings are the sites of attachment of the extra hydrogen atoms, as in the structures (V) and (VI) which have been previously suggested,[8,13] although without direct evidence. Structures such as (VII), containing adjacent dihydropyrrole rings, also require consideration since they have a molecular asymmetry which is consistent with the optical activity of stercobilin and because they too would give rise to hæmatinimide, succinic acid, and ethylmethylsuccinimide on oxidation.

Succinic acid obtained by oxidation of pyrophæophorbide-a is interpreted as derived from the β-carboxyethyldihydro-β′-methylpyrrole ring.[14] Succinic acid formation from stercobilin and d-urobilin (see below) cannot have the same significance because d-urobilin, which is a dehydro-i-urobilin, cannot contain hydropyrrole rings. This acid therefore probably arises from stercobilin and d-urobilin by partial destruction of the 3-carboxyethyl-4-methylpyrrole rings in these pigments. Structures such as (VII) are excluded by the absence of ethylmethylmaleimide and dihydrohæmatinimide in the oxidation products and by the production of a fairly high yield of hæmatinimide on the oxidation of stercobilinogen. In the oxidation of mesoporphyrin, hæmatinimide is difficult to isolate in high yield and the isolation of an amount approximately equivalent to one 3-carboxyethyl-4-methylpyrrole ring is considered to indicate the presence of two such rings per molecule of pigment. In the simple dipyrrylmethenes the introduction of a hydroxyl group into an α-position of the dipyrrylmethene group caused a hypsochromic shift of about 60—70 mμ in the main spectral band.[15] The much smaller difference between the positions of the main spectral bands of stercobilin and i-urobilin is inconsistent with the structure (VII) for stercobilin. The low yield of ethylmethylsuccinimide (maximum, 30%) obtained in our experiments, the extensive ring destruction obvious from the quantitative oxidations, and the quantity of pigment nitrogen converted into ammonia or amine may indicate contributions from structures such as (V) and (VI) in which the presence of isolated double bonds would reduce ring stability towards oxidation. In this case the end rings of stercobilin would probably constitute a complex tautomeric system composed of the four well-known individual systems (" 3-carbon," C=C–C–H; " keto-enol," C=C–O–H; " imino-enamine," C=C–N–H; and " amido-imidol," O=C–N–H [16]) the elements of which are discernible in the structures shown. It is possible, therefore, that the end rings of stercobilin, in common with prototropic systems in general, will vary according to their environment. However, in the absence of such tautomerism, ethylmethylsuccinimide could arise only from structure (VIII) which bears the extra hydrogen atoms in the

[12] Linstead and Whalley, *J.*, 1954, 3722.
[13] Siedel and Grams, *Z. physiol. Chem.*, 1940, **267**, 49.
[14] Linstead, Ficken, and Johns, *J.*, 1956, 2272.
[15] Pruckner and Dobeneck, *Z. phys. Chem.*, 1942, *A*, **190**, 43.
[16] Baker, " Tautomerism," Routledge, London, 1934.

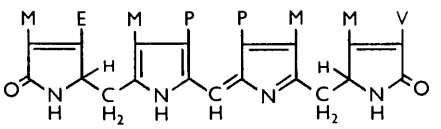
β-positions of the end rings; such a structure was first postulated by Birch,[17] and from the above considerations must be considered the most appropriate for stercobilin.

d-Urobilin

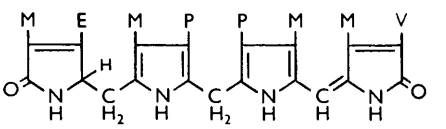
Oxidative Products of d-Urobilin.—d-Urobilin is usually obtained from infected fistula bile [18] or from the fæces of patients receiving antibiotic therapy,[5] but for the present investigation the material was isolated from the fæces of a patient with thalassemia and whose blood therefore contained a high proportion of fœtal hæmoglobin.

After oxidation of d-urobilin by 0·74N-chromium trioxide in sulphuric acid, hæmatinimide, succinic acid, and ethylmethylmaleimide were identified by paper chromatography. The last two substances were obtained crystalline and found to be identical with authentic compounds.

Catalytic Hydrogenation of d-Urobilin.—In methanol or acetic acid d-urobilin hydrochloride absorbed a maximum quantity of four equivalents of hydrogen per molecule in the presence of colloidal platinum. The *leuco*-compound thus obtained (tetrahydro-d-urobilin) was readily oxidised, by atmospheric oxygen or iodine, to give violet-brown solutions the dark colour of which made spectroscopic and polarimetric examination difficult; it was therefore impossible to assess accurately the amount of urobilin recovered and the optical activity retained. By avoiding prolonged solution of the pigment in chloroform it was possible to isolate as much as 12% of the original amount of d-urobilin as a crystalline dextrorotatory pigment. Quantitative hydrogenation showed that this pigment differed from the original d-urobilin, but resembled i-urobilin, in containing only one reducible double bond per molecule. Insufficient of this new compound was available for complete examination but the substance resembled d-urobilin in having an absorption maximum at 499 mμ in $CHCl_3$ and at 492 mμ in MeOH (extinction in the latter solvent being increased about three-fold on addition of free hydrogen chloride). If the hydrochloride is assumed to have an extinction coefficient equal to that of d-urobilin the pigment appears to have $[\alpha]_D$ +4950° in $CHCl_3$.



(IX)



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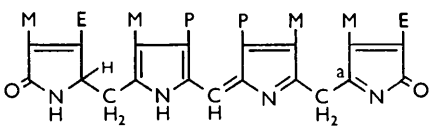
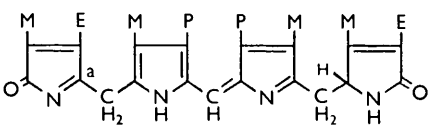


(XI)



(XII)

Treatment of d-urobilin or the new pigment with dilute aqueous alkali led to loss of optical activity and partial isomerism to violet pigments. Quantitative hydrogenation of the crystalline, racemised d-urobilin revealed the presence of two reducible double bonds per molecule.

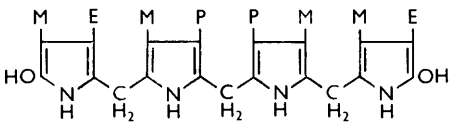
Pigments Obtained by Oxidation and Isomerisation of d-Urobilin.—Treatment of d-urobilin with excess of methanolic ferric chloride according to a method used by Legge for i-urobilin [19] caused only partial oxidation, up to 30% of the pigment being recovered as a urobilin. The products of this oxidation were separated into biliviolinoid and glaucobilinoid pigments by methods described in the Experimental section. The biliviolinoid pigments resemble those obtained similarly from i-urobilin.

[17] Birch, *Chem. and Ind.*, 1955, 652.
[18] Schwartz and Watson, *Proc. Soc. Exp. Biol. Med., N.Y.*, 1942, **49**, 641.
[19] Legge, *Biochem. J.*, 1949, **44**, 105.

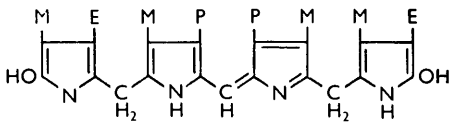
Violet pigments obtained by isomerisation in alkali of d-urobilin were separable into acid-, ether-, and chloroform-soluble fractions. The last-mentioned fraction contained most of the pigment as well as considerable unchanged urobilin. After removal of the urobilin the violet pigment showed an absorption maximum at 565 mμ in $CHCl_3$. The ethanolic zinc complex, with or without added iodine, was indistinguishable in its spectral properties from the same derivative of the products of oxidation.

The Structure of d-Urobilin.—The production of ethylmethylmaleimide by oxidation of d-urobilin indicates the presence of one or more 3-ethyl-4-methylpyrrole rings per molecule, provided that prototropic changes have not occurred. The absorption of four equivalents of hydrogen in the conversion of the pigment into a *leuco*-compound, presumably a dextrorotatory isomer of i-urobilin (see below), indicates that it is a dehydro-i-urobilin and this confirms Watson's conclusion [6] from elementary analyses. Because the dipyrrylmethene chromophore is unmodified the extra unsaturation in d-urobilin must be located either in the substituents in the 1-, 2-, 7-, or 8-position or in some nuclear position, for example, position (a) in (XI) and (XII), in which conjugation is impossible. The only obvious formulæ conforming to this requirement and to the available evidence are (IX) (or the isomer having interchanged ethyl and vinyl groups), (X), (XI), and (XII). Structures (XI) and (XII), because of the presence of one asymmetric carbon atom, could exhibit the optical activity shown by d-urobilin but could presumably arise only if biliverdin—which bears a hydrogen atom on each nitrogen atom—were an easily tautomerising substance. Structures such as (X) (α-hydroxydipyrrylmethenes in a pyrrolinone form) are improbable for the same reason that on spectroscopic grounds structure (VII) is inappropriate for stercobilin. Structures such as (IX) therefore appear to be the most probable for d-urobilin. The presence of a vinyl group, although not demonstrated experimentally, is considered more likely than that of an ethylidene group which has never been found in the bile pigments.

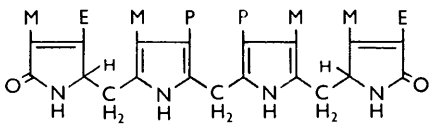
The isolation of a dextrorotatory pigment after mild oxidation of tetrahydro-d-urobilin was unexpected. If structure (IX) is correct for d-urobilin, tetrahydro-d-urobilin should be identical with mesobilirubinogen. This is a substance of known structure which forms i-urobilin on mild oxidation, and is formed on reduction of d-urobilin under conditions which favour racemisation [5] (*e.g.*, sodium amalgam or alkaline ferrous hydroxide). The usually accepted structures for mesobilirubinogen (XIII) and i-urobilin (XIV) imply the absence of any element of asymmetry apart from that which is inherent in the asymmetric IXα order of the β-side chains, and the absence of optical activity in these substances is



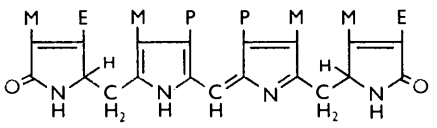
(XIII)



(XIV)



(XV)



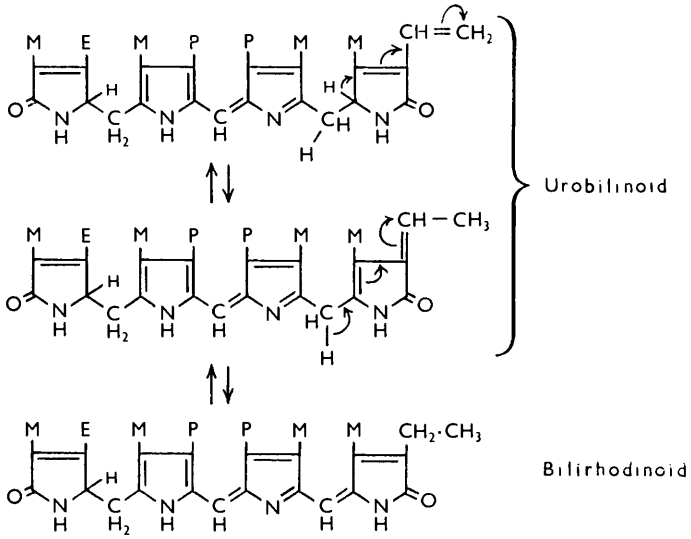
(XVI)

therefore consistent with such structures. However, the respective pyrrolinone and bislactam formulæ (XV and XVI) each contain two asymmetric carbon atoms per molecule and optical isomerism is then possible for mesobilirubinogen or for the urobilin of which it is the chromogen. We therefore suggest that the dextrorotatory urobilin obtained on oxidation of tetrahydro-d-urobilin is an optically active isomer of i-urobilin which would therefore be either an optically *meso*-compound or a racemic mixture of which the new

dextrorotatory urobilin is a component. The IXα-order of the β-side chains, confirmed analytically for d-urobilin and many of the other bile pigments,[2] must also obtain in the new pigment. We therefore suggest that this be called " d-urobilin-IXα " analogous to the name " urobilin-IXα " used for i-urobilin.

The conversion of d-Urobilin into Biliviolinoid Pigments.—Both i-urobilin and d-urobilin are readily dehydrogenated to biladienes and bilatrienes on treatment with ferric chloride. d-Urobilin appears to undergo this change under conditions in which oxidation is impossible. After treatment of an ethanolic solution of the hydrochloride with potassium *tert.*-butoxide, sodium hydroxide, or piperidine, addition of acetic acid produced a violet colour which was not obtained in control experiments in which addition of base was omitted. This colour reaction proceeded equally well when oxidation was excluded either by the passage of a stream of hydrogen or by the addition of sulphite. Even the strong reducing environment of tetrahydrofuran containing suspended lithium aluminium hydride failed to inhibit the production of violet pigment. Changes under these conditions cannot be oxidations and reduction could not lead to the formation of biliviolins. If the validity of structure (IX) for d-urobilin is assumed, simple tautomerisation to a mesobiliviolin or mesobilirhodin may be formulated as in Scheme *A*.

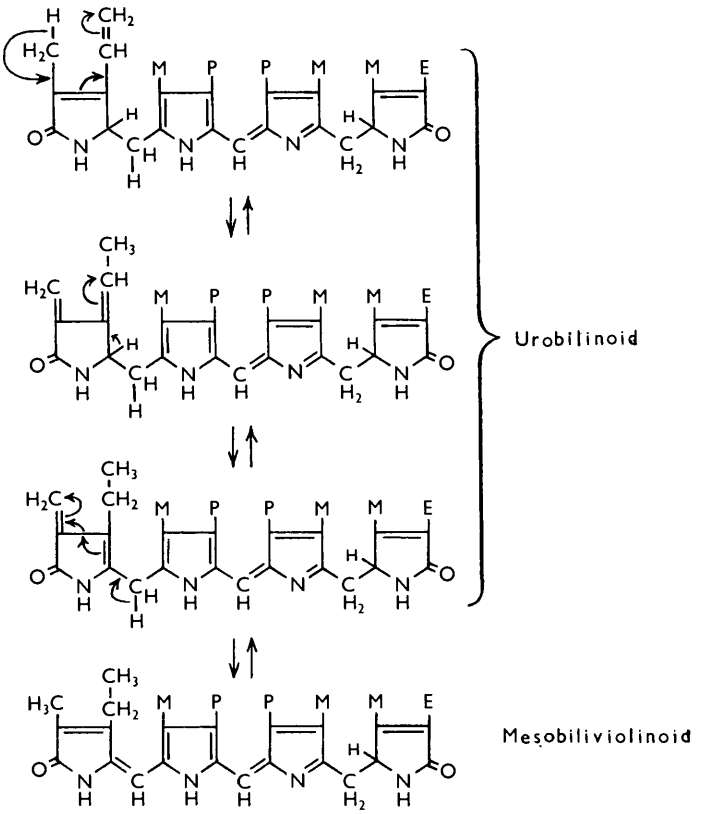
A



Similar changes, but involving hyperconjugation of the 1-methyl group, are possible if the vinyl group is assumed to be in the position 2 (Scheme *B*).

If compounds with structures (XI) and (XII) were found in Nature they would be capable of even simpler isomerisation to biliviolinoid pigments. If such modes of formation of biliviolin pigments by isomerisation are correct, these pigments should differ from those obtained by oxidation, for the latter would still contain one vinyl group. The relation between the two types of biliviolin might be somewhat comparable to that between the biliverdins and the mesobiliverdins, or between the biliverdins and the bilirubins and their dihydro-derivatives respectively. On treatment with iodine of the zinc complex of biliverdin the absorption band in the red is at 624 mμ. but that of mesobiliverdin is at 640 mμ.[20] However, the zinc complexes of mesobilirubin (with two ethyl groups) and of dihydrobilirubin (with one vinyl and one ethyl group), as well as of mesobiliverdin (with two ethyl groups) and dihydrobiliverdin (with one vinyl and one ethyl group), show no such spectroscopic difference after treatment with iodine. The same derivatives of the

[20] Plieninger, *Z. physiol. Chem.*, 1942, **274**, 231.



biliviolins obtained from d-urobilin have no such discrete bands in the red part of the spectrum, so that it was impossible to apply this method for distinction of the two types of biliviolin expected to arise.

Experimental

Microanalyses and quantitative micro-hydrogenations were carried out by Miss J. Cuckney and her colleagues at the Imperial College of Science. M. p.s were determined in the Kofler apparatus. Optical rotations were measured at 20—25°. Ultraviolet spectra were determined by using the Hilger spectrophotometer. Solvents were redistilled; and ether was freed from peroxide by treatment with ferrous sulphate solution. For column chromatography Savory and Moore's alumina, B.D.H. chromatographic calcium carbonate, and B.D.H. talc and magnesium oxide were used.

Paper Chromatography.—Descending development on Whatman No. 1 paper was used. The following solvents were used: (A) Xylene–phenol–85% formic acid (7 : 3 : 1, w/w/v) [21]; (B) ethanol–water–ammonia (*d* 0·88) (80 : 16 : 4 v/v/v); [22] (C) water-saturated ethyl acetate; (D) butan-1-ol–formic acid (95 : 5) saturated with water; [23] (E) pyridine–ammonia–water (6 : 2 : 1); [23] (F) butan-1-ol saturated with 1·5N-ammonia.[24]

Acidic compounds were located on the chromatograms by spraying the chromatograms with a solution of Bromocresol Green made just blue by addition of sodium hydroxide solution. Imides were detected by exposure to chlorine, aeration, and subsequent spraying with starch (0·25%) and potassium iodide (5%) solution. Chromatograms known to contain imides only were given the latter treatment without previous spraying with indicator. Authentic compounds were used as markers on the same paper.

[21] Kalbe, *ibid.*, 1954, **297**, 21.
[22] Long, Steadman, and Quale, *J.*, 1951, 2197.
[23] Linstead, Ficken, and Johns, *J.*, 1956, 2280.
[24] Brown and Hall, *Nature*, 1950, **67**, 166.

Reference Compounds.—Authentic *meso-* and DL-ethylmethylsuccinic acid were prepared and separated by methods similar to those used by Linstead and Whalley for the preparation of the αα′-dimethylsuccinic acids.[12] Dry distillation of the ammonium salt of the pure *meso*-acid always afforded a non-crystalline or poorly crystalline imide of poor elementary analysis, presumably a mixture of impure *meso-* and DL-ethylmethylsuccinimide. Similar treatment of the ammonium salt of the unpurified DL-acid gave a readily crystallisable imide (m. p. 56°) of good analysis for ethylmethylsuccinimide and this was assumed to be the pure DL-isomer.

Hæmatinimide and ethylmethylmaleimide were obtained by hydrolysis and oxidation of mesoporphyrin dimethyl ester by the method of Muir and Neuberger.[25] *meso-* and DL-Dihydrohæmatinic acids and DL-dihydrohæmatinimide were obtained according to the method of Linstead, Johns, and Ficken.[23]

Dihydrobilirubin and mesobilirubin were obtained by quantitative hydrogenation of bilirubin in 0·1N-sodium hydroxide in the presence of palladised charcoal. Glaucobilin, dihydrobiliverdin, and biliverdin were obtained in solution by oxidation of mesobilirubin, dihydrobilirubin, and bilirubin respectively with methanolic ferric chloride. The required verdins were extracted, first, into ether and then into 2·8N-hydrochloric acid from which they were extracted into chloroform.

Ethylmethylsuccinic Acid.—Ethyl α-bromopropionate (72 g.) was slowly added to a stirred solution of ethyl sodiomalonate prepared from sodium (9·2 g.), ethanol (150 ml.), and diethyl ethylmalonate (75 g.). After 6 hours' boiling, the ethanol was distilled off; the residue was dissolved in chloroform and washed free from halide. The residue was distilled, the fraction boiling at 165°/24 mm. being collected. This redistilled at 280—282°/760 mm. The product, a viscous syrup (35 g.), was again distilled and was hydrolysed in boiling 36% hydrochloric acid for 48 hr. On cooling, the solution deposited meso-*ethylmethylsuccinic acid* (7 g.) which was recrystallised to constant m. p. (187°) from water (Found: C, 52·6; H, 7·8. $C_7H_{12}O_4$ requires C, 52·4; H, 7·5%).

Ethylmethylsuccinimide from the meso-*Acid.*—The *meso*-acid (3·5 g.) was dissolved in ammonia (75 ml.; *d* 0·880), and the solution was evaporated almost to dryness. The residue was heated at 120° and the light yellow oil which distilled was discarded. Distillation was continued until, at 250°, a thick syrup distilled; this redistilled at 160—163°/25 mm.; it partially, but never wholly, crystallised (2·1 g.) (Found: C, 58·5; H, 8·0. $C_7H_{11}O_2N$ requires C, 59·5; H, 7·8%). The *product* in EtOH showed increasing light absorption from 270 to 220 mμ with an inflexion ($E^{1\%}_{1\,cm.}$ 3·8) at 248 mμ.

DL-*Ethylmethylsuccinimide.*—The mother-liquors remaining after the crystallisation of the *meso*-acid were exhaustively extracted with ethyl acetate. Evaporation of the dried extract gave a non-crystallisable syrup. Concentrated ammonia solution was added until the mixture was alkaline and the solution was diluted, with water, to 100 ml. A small quantity of unhydrolysed ester was extracted into chloroform, and the syrup obtained by evaporation of the whole solution was dry-distilled at 130—280°/760 mm. Crystallisation from water furnished a *product* of m. p. 54—55° (2·3 g.) (Found: C, 59·4; H, 8·0; N, 9·8. $C_7H_{11}O_2N$ requires C, 59·5; H, 7·8; N, 9·9%), showing increasing absorption in EtOH from 270 to 220 mμ with one inflexion ($E^{1\%}_{1\,cm.}$ 6·2) at 248 mμ.

meso*Dihydrohæmatinic Acid.*—Sodium (6·2 g.) was dissolved in ethanol (200 ml.). To the cooled, stirred solution ethyl cyanoacetate (30 g.) was added. About a third of the alcohol was removed, and ethyl α-bromopropionate (48 g.) was slowly added with stirring. When deposition of sodium bromide had ended the mixture was boiled, under reflux, until no longer alkaline. Volatile material was distilled off and water was added to the residue. The organic fraction was extracted into ether and washed free from halide. After drying (Na_2SO_4) and removal of ether the residue was distilled, the fraction of b. p. 159°/30 mm. (26 g.) being collected. The yield of redistilled diethyl α-cyano-α′-methylsuccinate, b. p. 156°/20 mm., was 20 g.

Sodium (2·2 g.), dissolved in ethanol (30 ml.), was quickly added to a stirred solution of the diethyl α-cyano-α′-methylsuccinate (20 g.) in ethanol (30 ml.). Ethyl β-bromopropionate (25 g.) was slowly added and the mixture was heated at 90° for 10 hr., then being neutral to litmus. Volatile solvent was removed and the ester separated in the way described above. A small amount of unchanged ethyl β-bromopropionate was removed and the residue was distilled, giving a fraction of b. p. 220°/38 mm. (19 g.). The product was hydrolysed by boiling with 36% hydrochloric acid (200 ml.) for 20 hr. A small quantity of unhydrolysed ester was

[25] Muir and Neuberger, *Biochem. J.*, 1949, **45**, 164.

separated from the hot mixture which was then concentrated to 50 ml. and cooled. The *meso*-acid which crystallised at this stage was recrystallised from acetone to constant m. p. (176—178°) (4 g.; a further quantity was recovered from mother-liquors—see below) (Found: C, 47·1; H, 5·9. Calc. for $C_8H_{12}O_6$: C, 47·0; H, 5·9%).

DL-*Dihydrohæmatinic Acid.*—The mother-liquor from the preparation of the *meso*-acid was continuously extracted with ether for several days; this solution, on cooling, deposited 2·5 g. of acid, m. p. 141°, which recrystallised from acetone to the correct m. p. for the *meso*-acid, bringing the total yield of that isomer to 6 g. Evaporation of the ethereal solution afforded a solid which was heated for 3 hr. at 200°. The product was boiled with dilute hydrochloric acid and charcoal, filtered, and evaporated to dryness. The residue was recrystallised from acetone to constant m. p. (144°) (0·9 g.) (Found: C, 47·1; H, 5·9%).

DL-*Dihydrohæmatinimide.*[23]—*meso*-Dihydrohæmatinic acid (2·2 g.) and urea (0·81 g.) were fused together at 185° until no more carbon dioxide was evolved (1 hr.). The cooled mass dissolved in water was acidified with 36% hydrochloric acid (5 ml.), and the imide was extracted continuously into ethyl acetate. The extract was washed with a small quantity of water and filtered. The syrup obtained by evaporation of the ethyl acetate was redissolved in fresh solvent and warmed with benzylamine (1 ml.). The precipitated benzylamine salt was recrystallised to constant m. p. (153°) from methyl alcohol–ethyl acetate (yield 0·5 g.). The benzylamine salt was dissolved in cold water (20 ml.) and the solution was acidified. The liberated imide was extracted into ether and washed with a little water. Evaporation of the solvent left a syrup which slowly crystallised *in vacuo* over sulphuric acid (m. p. 85—86°) (Found: N, 7·5. Calc. for $C_8H_{11}O_4N$: N, 7·6%).

Isolation of Stercobilin Hydrochloride. (*Watson's method* [11] *with minor modifications*).—Fæces were exhaustively extracted with ethereal acetic acid. The residue, left after distillation of the extract, was poured into 6 volumes of 1% w/v hydrochloric acid, and left for 16 hr. After filtration from fat and neutralisation with potassium acetate, porphyrins were removed by ether. Stercobilin was then removed from the aqueous solution by exhaustive extraction with chloroform and was re-extracted from the chloroform into water. The free urobilin in aqueous solution was converted into the hydrochloride by addition of 9% v/v hydrochloric acid and was again extracted into chloroform. The concentrated solution of stercobilin, obtained by almost complete evaporation of the chloroform solution, was poured dropwise into light petroleum, and the precipitated pigment was crystallised from cold, dry acetone. Further purification to high optical activity was achieved by recrystallisation from chloroform (containing a trace of methanol in the later stages) and then from methanol–ethyl acetate. This procedure provided a product of $[\alpha]_D$ −3000° or less (in $CHCl_3$) and this was increased by drying the substance over phosphoric oxide *in vacuo* at 60—70°. Yields ranged from 7 to 130 mg. of hydrochloride per 100 g. of moist fæces, $[\alpha]_D$ ranging from −3150° to −3850° in $CHCl_3$ (lit.,[5] −4000°). Fæces from normal subjects rarely afforded worthwhile quantities of pigment; considerably larger yields were obtained from the fæces of patients with hæmolytic disease.

Isolation of d-Urobilin.—The method outlined for stercobilin was followed with fæces from a subject with thalassæmia, but much less ether was used for the extraction of porphyrins and the solution, after the precipitation of fats, was worked up after only 30 min. In later preparations 10—20 ml. of 1% iodine solution were added at this stage to increase the rate of conversion of the extracted d-urobilinogen into d-urobilin. Storage overnight as in the stercobilin preparations gave much violet pigment with less urobilin and more difficulty in purification. When fairly pure, d-urobilin hydrochloride is less soluble in chloroform than is stercobilin hydrochloride and in the later stages of recrystallisation it was necessary to incorporate about 10% (v/v) of methanol in the chloroform. The added methanol was partly removed by evaporation of the filtered solution whilst fresh chloroform was continuously added. Crystallisation continued easily when the solution was cooled at the first sign of deposition of pigment. The product so obtained was finally freed from occluded chloroform by crystallisation from methanol–ethyl acetate and was dried in the same way as stercobilin but at 35—40°. Yields ranged from 10 to 50 mg. of hydrochloride per 100 g. of moist fæces and $[\alpha]_D$ from +4100° to +4700° (lit.,[6] +5000°).

Examination of chloroform solutions containing 1·35—0·13 mg. of urobilin hydrochloride per 100 ml. gave $E^{1\%}_{1\,cm.}$ 1450 at 499 mμ.

25·79 mg. of hydrochloride absorbed 1·82 ml. of hydrogen at N.T.P. (theor. for 2 double bonds 1·85 ml. at N.T.P.). 29·50 mg. absorbed 2·09 ml. of hydrogen at N.T.P. (theor. 2·12 ml.).

Quantitative Experiments on the Oxidation of Stercobilin Hydrochloride.—Crystalline stercobilin hydrochloride was suspended in 50% (v/v) aqueous sulphuric acid at 0° and an equal volume of 1·48N-chromium trioxide solution was slowly added. The reaction was allowed to proceed at room temperature for 64—67 hr. Evolved carbon dioxide was aspirated, in a stream of nitrogen, into tubes containing 2N-sodium hydroxide or N-barium hydroxide. In the former case barium carbonate was precipitated by the addition of 20% barium chloride solution, washed, dried, and weighed; in the latter, residual barium hydroxide was titrated, in the presence of acetone, with standard acid.[25] The reaction mixture was diluted and residual oxidant was estimated iodometrically. The results were calculated as atoms of carbon, per molecule of pigment, converted into carbon dioxide and equivalents of oxidation consumed. These results are in the Table.

Stercobilin hydrochloride used (mg.)	$[\alpha]_D$ ($CHCl_3$)	Equivs. of oxidant added per mol.	C atoms/mol. oxid. to CO_2	Equivs. oxidant consumed
26·5	−3340°	49·3	—	38·6
23·0	−3773	56·8	4·8	38·2
20·0	−3773	75·0	5·6	39·5
22·0	−3773	67·8	5·3	38·2
22·0	−3773	42·5	4·8	37·5
100·0	−3335	36·6	4·4	33·0

Oxidation of Stercobilin Hydrochloride with Isolation of Succinic Acid and Ethylmethylsuccinimide.—Stercobilin hydrochloride (225 mg., $[\alpha]_D$ −3850° in $CHCl_3$), suspended in 50% (v/v) aqueous sulphuric acid (8·75 ml.), was stirred at −5° during the slow addition of 1·48N-aqueous chromium trioxide (8·75 ml., 36 equivs. per mol.). Stirring was continued for 6 hr. during which the solution was allowed to attain room temperature. After 48 hr. residual oxidant was destroyed by sodium hydrogen sulphite, and volatile material was aspirated into 2 : 4-dinitrophenylhydrazine solution by a stream of nitrogen; no precipitate of phenylhydrazone was produced. The reaction mixture was diluted with water (10 ml.), and the products of oxidation were removed in two main fractions by extraction, first with chloroform (3 × 30 ml.), then with ethyl acetate (5 × 30 ml.). Paper chromatography of the chloroform extract at this stage, with solvent F, revealed an acidic substance R_F 0·12 corresponding to formic acid and acetic acid which are not separable in this system. The compound was assumed to be acetic acid because of its failure to reduce ammoniacal silver nitrate solution. Each extract was washed twice with water (2 ml.), dried by filtration, and evaporated. The original aqueous solution then contained 7·4 mg. of nitrogen (*i.e.*, 37% of the nitrogen originally present in the stercobilin). Continuous ether-extraction removed a minute quantity of syrup which contained succinic acid (R_F 0·12—0·13 in solvent A), leaving in the aqueous phase 4·97 mg. of nitrogen of which 4·34 mg. were present as ammonia or a volatile amine, displaceable by boiling alkali. Evaporation of the ethyl acetate extract afforded a semicrystalline mass (108·6 mg.) which dissolved completely in acetone (2 ml.). 0·01 ml. quantities of the solution were used for paper chromatography with the following results. In solvent E: R_F, 0·11 (acid) not identified, 0·22 (acid) (cf. succinic acid R_F 0·20), 0·45 (acid imide) faint and unidentified, 0·60 (acid imide) (cf. hæmatinimide R_F 0·62), and 0·79 (imide) (cf. DL-ethylmethylsuccinimide R_F 0·77). In solvent A: R_F 0·10 (acid) (cf. succinic acid R_F 0·10), 0·36 (acid imide) (cf. hæmatinimide R_F 0·36), 0·54 (acid imide) faint and unidentified, and 0·80 (imide) (cf. DL-ethylmethylsuccinimide R_F 0·78). After removal of the acetone the products from the ethyl acetate extract were dissolved in water (10 ml.), a small quantity of insoluble succinic acid (m. p. and mixed m. p.) was removed, and the solution was made alkaline by N-sodium hydroxide (2 ml.). Ether-extraction at this stage removed no material. The solution was brought, successively, to pH 6, 4, and 2 by addition of hydrochloric acid, with extraction at each stage with ether (100 ml.). Extraction at pH 6 removed a yellow oil (13·5 mg.) which did not crystallise but contained hæmatinimide (R_F 0·85 in solvent C, 0·48 in solvent B, and 0·56 in solvent E). The semicrystalline materials obtained by evaporation of the extracts of pH 2 and 4 (29·2 and 50·8 mg. respectively) were shown to be identical by paper chromatography. They were united and resolved, on silica gel in ether, into two main fractions: succinic acid (total 12·6 mg.) (m. p. and mixed m. p.) and a minor imide fraction (R_F 0·48 in solvent B and 0·85 in solvent C, corresponding to hæmatinimide).

Evaporation of the chloroform extract left a clear oil (66·4 mg.). This in fresh chloroform (0·5 ml.) exhibited a slight positive rotation in sodium light (0·2° in a 0·5 dm. tube). Paper

chromatography of the solution gave the following results. Solvent E: R_F 0·20 (acid) probably succinic acid, 0·57 (acid imide) (cf. hæmatinimide R_F 0·62), 0·71 (imide) (cf. DL-ethylmethylsuccinimide R_F 0·77). Solvent D: R_F 0·75 (imide) (cf. DL-ethylmethylsuccinimide R_F 0·75). Solvent A: R_F 0·30 (acid imide) (cf. hæmatinimide R_F 0·30), 0·60 (acid imide) faint and unidentified, 0·78 (imide) (cf. DL-ethylmethylsuccinimide R_F 0·78). The oil was dissolved in benzene (2 ml.) and eluted with the same solvent from a column of silica gel (water-saturated and allowed to dry in air). All fractions containing imide were united and on evaporation gave 25·8 mg. of crude imide. This was distilled in a micro-sublimation apparatus at 13—17 mm. The middle fraction (17 mg.) was twice redistilled, to give a straw-coloured oil (13·9 mg.) which did not crystallise even when seeded with DL-ethylmethylsuccinimide (Found: C, 59·5; H, 7·9. Calc. for $C_7H_{11}O_2N$: C, 59·5; H, 7·8%).

Identification of the Natural Imide.—The neutral, chloroform-soluble syrup obtained in the above experiment and authentic DL-ethylmethylsuccinimide in methanol (10^{-4} g. in 0·02 ml.) were used for chromatography in solvent B. The natural product gave spots of R_F 0·78 and 0·73 (two chromatograms) corresponding to R_F 0·78 and 0·79 for the authentic DL-imide. A mixture of authentic and the natural imide gave a single spot.

Both the oxidation product and authentic imide in ethanol had absorption maxima below 220 mμ, with an inflexion at 248 mμ ($E^{1\%}_{1cm.}$ 7·5 and 6·2 respectively). The curve for the oxidation product had also a slight inflexion at 280 mμ ($E^{1\%}_{1cm.}$ 1·8). Chloroform solutions of the natural and the authentic imide and the product of dry distillation of ammonium *meso*-ethylmethylsuccinate had infrared maxima at 1780 s, 1725 vs, 1458 m, 1382 m, 1347 s, 1286 m, and 1172 s, $cm.^{-1}$.

The above three compounds (4 mg. each) were heated (80°; 2 hr.) with concentrated hydrochloric acid (0·5 ml. for each) in sealed tubes, then diluted with water (0·5 ml.) and extracted with ether (10 ml.). The ethereal solutions were filtered and evaporated, and each residue was dissolved in ethanol (0·2 ml.); used for paper chromatography with solvent A, each gave one acid spot (R_F 0·47) corresponding to R_F 0·47 for DL- and *meso*-ethylmethylsuccinic acid.

Attempt to detect Dihydrohæmatinimide after Oxidation of Stercobilin Hydrochloride.—The ethyl acetate extract of the oxidation products of stercobilin hydrochloride (27 mg.) with 1·48N-chromium trioxide solution (1·05 ml.) and 50% v/v aqueous sulphuric acid (1·05 ml.) was used in methanol (1 ml.) for paper-chromatographic comparison with hæmatinimide (0·052 mg. in 0·02 ml. of methanol; R_F 0·85) and DL-dihydrohæmatinimide (same concn., R_F 0·71) with solvent C. Spots at the correct position for hæmatinimide and succinic acid were obtained and the mixture of products of oxidation and DL-dihydrohæmatinimide gave double spots (R_F 0·78, 0·70). In the same system DL-dihydrohæmatinic acid had R_F 0·54.

Oxidation of Stercobilinogen.—Stercobilin hydrochloride (50 mg.; $[\alpha]_D$ −3700° in $CHCl_3$) in 0·5N-sodium hydroxide (10 ml.) was reduced with hydrogen in the presence of colloidal palladium. After acidification with sulphuric acid the solution was extracted with light petroleum (250 ml.). The extract was washed with water (2 × 10 ml.), filtered, and evaporated. N-Chromium trioxide (3·2 ml., *ca.* 40 equiv.) and 20% hydrochloric acid (5 ml.) were added to the residue. The solution was kept at 20° for 15 hr., diluted with water (20 ml.), and extracted with chloroform. Spectroscopic examination of the diluted chloroform extract showed the presence of *ca.* 6·4 mg. of unchanged urobilin. The residue given by evaporation of the chloroform extract was dissolved in ethyl acetate. The original aqueous solution was extracted with the same solvent (100 ml.), and the two solutions were united. The residue remaining after the evaporation of the ethyl acetate was dissolved in water (10 ml.) and adjusted to pH 7 by sodium hydrogen carbonate. Extraction with chloroform at this stage removed a small amount of yellow material but evaporation of the extract left a negligible residue. The aqueous solution was brought to pH 5 by hydrochloric acid, and acetate buffer (pH 5·2) was added. Extraction with ethyl acetate then removed a syrup which was chromatographed in methanol (0·5 ml.) on Whatman No. 1 paper with water-saturated ethyl acetate; the appropriate section of the paper (R_F 0·8) was eluted with methanol, and the solution was evaporated to a syrup which crystallised after storage over sulphuric acid and seeding with authentic hæmatinimide (m. p. and mixed m. p. 118°; 12 mg.).

Oxidation of d-Urobilin Hydrochloride.—To d-urobilin hydrochloride (67 mg.; $[\alpha]_D$ +4520° in $CHCl_3$), suspended in 50% v/v aqueous sulphuric acid (2·6 ml.) at 0°, was added 1·48N-chromium trioxide solution (2·6 ml.). The mixture was kept at 20° for 44 hr., then treated with a trace of sodium hydrogen sulphite and extracted with chloroform (4 × 10 ml.). The

extract was washed with water (2 ml.), which was added to the original aqueous solution. The latter was then extracted with ethyl acetate (4 × 10 ml.), and the extract was washed as before. The chloroform and ethyl acetate solutions were dried by filtration and evaporated at 20°. The residual syrup from the chloroform extract crystallised when seeded with ethylmethylmaleimide (m. p. 58—60°) (11 mg., 36·7% of the yield possible if two 3-ethyl-4-methylpyrrole rings are present per molecule). The crystalline product was cautiously sublimed and crystallised from water, to give 8·6 mg. of m. p. 65° (mixed m. p. 66°). The infrared characteristics of the oxidation product and of authentic imide in potassium chloride were identical: 1765 s, 1710 s, 1455 m, 1385 m, 1350 s, 1175 m, 1065 m, 1055 m, 945 w, 735 m, cm.$^{-1}$. In spite of this identity the oxidation product appeared to contain small amounts of hæmatinimide and succinic acid (see paper chromatography below).

The residue obtained from the ethyl acetate extract was dried at 40° *in vacuo*, giving white semicrystalline material. A little chloroform at 0° removed a slightly dextrorotatory syrup (16·6 mg.) and the residual solid was sublimed at 13 mm. (m. p. 180—182° alone or mixed with succinic acid) (8·6 mg., 34% of the yield for complete destruction of two 3-carboxyl-4-methylpyrrole per molecule of pigment). Continuous ether-extraction of the original aqueous solution removed no further product, and the aqueous solution then contained 2·34 mg. of nitrogen (39% of the original pigment-nitrogen) of which 2·1 mg. (35% of the original nitrogen) were displaceable as ammonia or amine by boiling alkali.

The ethylmethylmaleimide (A) (8·6 mg.), the residue (B) (16·6 mg.) obtained by evaporation of the chloroform-washings of the ethyl acetate-soluble substance, and the chloroform-insoluble product (C) (8·6 mg.) were dissolved in methanol (1·72, 3·2, and 1·72 ml. respectively); 0·02 ml. of each solution was used for paper-chromatography in solvent C with the following results. A, spots at R_F 0·90 (cf. ethylmethylmaleimide 0·90) 0·40 (cf. succinic acid 0·40) (faint), and 0·78 (cf. hæmatinimide 0·78) (faint). B, spots at R_F 0·38 (cf. succinic acid 0·40), 0·78 (cf. hæmatinimide 0·76), and 0·15 unidentified. C, R_F 0·43 (cf. succinic acid 0·43).

Catalytic Reduction of d-Urobilin Hydrochloride and Isolation of d-Urobilin-IXα.—d-Urobilin hydrochloride (105·3 mg., $[\alpha]_D$ +4390° in $CHCl_3$) in acetic acid (10 ml.) in the presence of Adams platinum (33 mg.) absorbed 7·9 ml. (N.T.P.; 2 mols.) of hydrogen. The solution was then diluted with water (75 ml.), treated with 0·1N-iodine (2 ml.), and shaken with successive small quantities of chloroform until no further colour was removed; a further 1 ml. of iodine solution was added to the aqueous phase, and the chloroform extraction was repeated. Addition of iodine and extraction was continued until conversion of the *leuco*-compound into urobilin and extraction of the latter were complete. The aqueous solution finally gave no reaction with Ehrlich's reagent. Each successive portion of chloroform extract obtained was at once extracted with water (*i.e.*, the urobilin was removed as soon as possible from the chloroform in which it appeared to undergo rapid change to deeply coloured compounds). To the aqueous solution of free pigment (800 ml.), concentrated hydrochloric acid (72 ml.) was added and all urobilin hydrochloride was extracted into chloroform (500 ml.). The solution was dried (Na_2SO_4) for a short time and filtered. Spectroscopic examination of the chloroform solution showed the presence of 15 mg. of urobilin of $[\alpha]_D$ +4900° in $CHCl_3$ (pigment assumed to have the same extinction coefficient as d-urobilin hydrochloride). The crude urobilin precipitated from concentrated chloroform solution by the addition of cold dry acetone was recrystallised twice from methanol–ethyl acetate (yield 12 mg., dried at 35° *in vacuo* over P_2O_5). The residue given by evaporation of the mother-liquors from the precipitation and crystallisation of the above product was dissolved in saturated sodium acetate solution (4 ml.) and brought on to a column of alumina in water. Elution with water removed an orange band containing a further 0·6 mg. of urobilin, leaving all violet pigment at the head of the column (total yield, 12% of original urobilin). Comparable amounts of the hydrochlorides of d-urobilin, stercobilin, and d-urobilin-IXα were submitted for hydrogenation with the following results.

Pigment	Quantity hydrogenated (mg.)	H_2 absorbed (ml. at N.T.P.)	C:C present	C:C indicated
d-Urobilin	11·76	0·841	2	2
Stercobilin	12·23	0·447	1	1
d-Urobilin-IXα	11·33	0·416	?	1

Two further experiments afforded d-urobilin-IXα.

(*a*) d-Urobilin hydrochloride (55·3 mg.: $[\alpha]_D$ +4220° in $CHCl_3$), reduced catalytically in acetic acid and allowed to reoxidise spontaneously, gave 6·9 mg. (12%) of recovered urobilin.

The chloroform solution had $\lambda_{max.}$ 499 mμ. The methanol solution had $\lambda_{max.}$ 492 and 365 mμ, intensity at the former being increased about three-fold on addition of hydrochloric acid. The crystalline hydrochloride had $[\alpha]_D$ 4250° in $CHCl_3$. The mother-liquors contained violet pigment with $\lambda_{max.}$ at 560—570 mμ.

(*b*) The hydrochloride (50 mg.; $[\alpha]_D$ +4700° in $CHCl_3$), reduced in methanol and reoxidised with iodine, gave 16·9 mg. (34%, estimated spectroscopically) of recovered urobilin of which 5·6 mg. (10·4%) were isolated as the crystalline hydrochloride of $[\alpha]_D$ +3200° in $CHCl_3$).

Racemisation of d-Urobilin.—To d-urobilin hydrochloride (50 mg.; $[\alpha]_D$ +4400° in $CHCl_3$) in water (4 ml.) 2·5N-sodium hydroxide (4 ml.) was added. After 45 min. under hydrogen the solution was diluted to 200 ml. and acidified with 2·5N-hydrochloric acid (8 ml.). All orange pigment was then removed by chloroform extraction, leaving the aqueous solution slightly violet. The chloroform extract, dried by filtration and diluted to 250 ml., had $\lambda_{max.}$ at 499 mμ and, $E^{1\%}_{1cm.}$ being assumed equal to that of d-urobilin, appeared to contain 31 mg. of urobilin. This solution was optically inactive (for $[\alpha]_D$ +4000° in $CHCl_3$ the theoretical rotation would be 1°). Crystalline racemic urobilin was obtained by precipitation of the crude pigment in acetone and crystallisation twice from chloroform and once from methanol–ethyl acetate (yield, 14 mg. after drying at 28° over P_2O_5 in a vacuum).

The residue left on evaporation of the mother-liquors was dissolved in saturated sodium acetate solution (4 ml.) and brought on to a column of alumina in water. Elution with water separated a yellow orange band which contained a further 3 mg. of racemic urobilin. Examination of concentrated solutions of either of the two fractions failed to reveal any optical activity.

14·94 mg. of the product absorbed 1·05 ml. (N.T.P.) of hydrogen, the second mol. more slowly (theor. for 2 C:C, 1·07 ml.).

Production of Biliviolinoid Pigments by the Isomerisation and Oxidation of d-Urobilin.—(a) *Oxidation.* d-Urobilin hydrochloride (2·5 mg.; $[\alpha]_D$ +4390° in $CHCl_3$) and a 20% ferric chloride solution in 36% hydrochloric acid (0·5 ml.) in methanol (5 ml.) were boiled for 15 min., diluted with water (25 ml.), rendered neutral to Congo-red by saturated sodium acetate solution (4 ml.), and extracted with ether (150 ml.). Unoxidised urobilin was removed from the ethereal solution by water (8 × 5 ml.), these washings being added to the original aqueous solution. Most of the violet pigment was extracted from the ethereal solution, with considerable deepening of colour, by 2·8N-hydrochloric acid (12 × 5 ml.). The acid solution, diluted to 100 ml. with 2·8N-hydrochloric acid, had $\lambda_{max.}$ at 560—570 mμ (optical density 0·54). Concentrated hydrochloric acid (5 ml.) was added to the original aqueous solution, and urobilin was extracted into chloroform (2 × 50 ml.); spectroscopic examination of this extract showed the presence of 0·7 mg. (30%) of urobilin which, again treated as above, underwent 70% oxidation to violinoid pigments. Violet pigments from the second oxidation in chloroform (20 ml.) showed absorption maxima at 564 mμ (optical density 1·0) with a shoulder at 585—605 (optical density 0·90 at 595 mμ) and 500 mμ (optical density 0·64). The residue left on evaporation of the chloroform solution, dissolved in saturated ethanolic zinc acetate (30 ml.), had absorption maxima at 630 (optical density 0·85), 580 (optical density 0·41) and 510 mμ (optical density 0·33). Addition of 1 drop of 1% ethanolic iodine caused intensification of the maximum at 510 mμ (optical density, 1·16) and almost complete disappearance of the maxima at 580 and 630 mμ (optical density 0·06 at both).

The biliviolinoid pigments obtained by similar oxidation of 5·3 mg. of d-urobilin hydrochloride were eluted from talc with chloroform–ether (2 : 1 v/v). From the main violet band a minor green band partially separated. That portion of the chromatogram containing pure green pigment was removed and the pigment was washed out into chloroform–methanol (1 : 1 v/v). Impure violet pigment was removed from the rest of the column in the same way; the solution was evaporated, and the pigment was dissolved in chloroform–ether (1 : 1 v/v; 2 ml.), and was eluted with the same solvent through a column of magnesium oxide. Some violet and green pigments remained, in separate zones, at the head of the column. A violet band migrated and separated into a (lower) reddish-violet band and a (minor) blue band. A chloroform solution of the pure green pigment showed a broad absorption maximum at 610—670 mμ, the curve being similar to that of glaucobilin in the same solvent but with enhanced absorption at 640—680 mμ. The zinc complex salt, oxidised with ethanolic iodine, showed maxima at 620—625, 575, and 510 mμ. Glaucobilin, dihydrobiliverdin, and biliverdin, treated similarly, gave solutions with the last two maxima at the same position and with the first band at 625, 630—635, and 630—635 mμ, respectively. A chloroform solution of the violet

pigment showed an indistinct maximum at 560—575 mμ with a pronounced shoulder at 590—610 mμ. The latter characteristic disappeared when the solution was washed with water, the absorption curve then showing a fairly broad maximum at about 560 mμ.

(b) *Isomerisation.* d-Urobilin hydrochloride (5·3 mg.; $[\alpha]_D$ +4390° in $CHCl_3$) in methanol (5 ml.) was treated with 2N-sodium hydroxide (1 ml.) and boiled for 18 min. in an atmosphere of hydrogen. To the cooled solution water (25 ml.), saturated sodium acetate solution (4 ml.), and acetic acid (10 ml.) were added. There was a change of colour from brown to violet and all pigment was extracted into ether (4 × 50 ml.). The amber aqueous solution then contained 0·7 mg. of residual urobilin. Extraction of the ethereal solution with water (16 × 5 ml.) removed a reddish-violet pigment, the solution having $\lambda_{max.}$ 555—558 mμ (unchanged on acidification). Pigment remaining in the ether was extracted with 2·8N-hydrochloric acid (23 × 5 ml.) with change of colour from reddish-violet to pure violet. The two pigment fractions were extracted from aqueous solution into chloroform (70 ml. in each case); the chloroform solutions were washed with water (10 ml.), dried by filtration, and diluted to 100 ml. The chloroform solution of the pigment initially extracted into hydrochloric acid showed absorption maxima at 573—575 mμ. Both chloroform solutions showed slight maxima at 499—500 mμ, due to the presence of a little urobilin; they were united and evaporated to dryness. The residual pigments were dissolved in saturated sodium acetate solution (4 ml.) containing a little acetic acid, diluted with water (25 ml.), and extracted with ether (4 × 50 ml.). The ethereal solution was washed free from urobilin with water (5 × 10 ml.), and all violet pigment was extracted (with deepening of colour) into 2·8N-hydrochloric acid (20 × 5 ml.). The pigment was extracted from acid solution into chloroform (80 ml.). The chloroform solution was washed with water (20 ml.) and dried by filtration; it then had absorption max. only at 577 mμ. A concentrated solution of the zinc complex salt in ethanol showed, after oxidation with iodine, an indistinct and weak maximum at 618—620 mμ.

Other Isomerisation Experiments giving Biliviolinoid Pigments.—(*a*) d-Urobilin hydrochloride (2 mg.) in methanol (7 ml.) containing a few crystals of sodium sulphite was warmed, under hydrogen, at 60° for 5 min. with piperidine (2 ml.), 2·5N-sodium hydroxide (2·0 ml.), or butan-1-olic potassium *tert.*-butoxide (2% of potassium; 0·5 ml.). Addition of excess of acetic acid to the cooled solution produced a brilliant violet colour. No colour developed in solutions treated similarly but without the addition of base.

(*b*) To d-urobilin hydrochloride (20 mg.; $[\alpha]_D$ +4300° in $CHCl_3$) in methanol (20 ml.) under hydrogen 2N-sodium hydroxide solution (4 ml.) was added. The solution was heated under hydrogen at 66° for 18 min., gradually deepening in colour, finally to violet; this colour persisted on dilution to 200 ml. and was intensified on addition of acetic acid (5 ml.). Most pigment was extracted from the acid solution by ether (350 ml.) and was re-extracted from ether into water. Small quantities of pigments remaining in the original aqueous and ethereal solutions were extracted into chloroform (116 ml.) [$\lambda_{max.}$ 499 mμ (optical density, *D* 1·62), 570 mμ (*D* 0·21)] and 10% hydrochloric acid respectively. The pigments in 10% hydrochloric acid were extracted into chloroform (174 ml.) [$\lambda_{max.}$ 505 (*D* 0·34), 570 (*D* 0·29); $\lambda_{min.}$ 535 mμ (*D* 0·21)]. The water-soluble pigments extracted from ether solution were extracted into chloroform (230 ml.) [$\lambda_{max.}$ 505 (*D* 0·85), 720 (*D* 0·40); $\lambda_{min.}$ 555 mμ (*D* 0·38)]. After extraction with 10% hydrochloric acid the ethereal solution was pale green but slowly became violet; the pigment was extracted into 10% hydrochloric acid (again leaving a solution of labile green pigments), and the pigment was extracted from the acid solution into chloroform (64 ml.) [$\lambda_{max.}$ 560—570 mμ (*D* 0·17)].

(*c*) d-Urobilin (16·3 mg.; $[\alpha]_D$ +4320° in $CHCl_3$) in freshly distilled, peroxide-free tetrahydrofuran (5 ml.) was boiled with lithium aluminium hydride (100 mg.) for 1½ hr., becoming violet. After being kept overnight (under hydrogen) excess of hydride was destroyed by dilute hydrochloric acid, the solution diluted with water (300 ml.), and concentrated hydrochloric acid (10 ml.) added. Most of the pigment was extracted into chloroform (400 ml.); the chloroform solution was evaporated to dryness and the residue was dissolved in 10% potassium acetate solution (130 ml.). The aqueous solution was extracted with ether (200 ml.) which removed a small quantity of violet pigment. Re-extraction of the ethereal solution with 1·5N-hydrochloric acid removed the violet pigment, leaving the ethereal solution greenish-yellow. The ether was evaporated to provide a green residue which gave a solution in chloroform (100 ml.) with $\lambda_{max.}$ 410 mμ (*D* 0·143). Pigment remaining in the aqueous solution after the ether-extraction was extracted into chloroform (500 ml.) giving a reddish solution with

$\lambda_{max.}$ 570 mμ (*D* 1·066). The aqueous solution (140 ml.), now of urobilinoid colour with $\lambda_{max.}$ 492 mμ (*D* 0·65), was treated with concentrated hydrochloric acid (20 ml.). All urobilin was then extracted into chloroform (150 ml.); the resulting solution had $\lambda_{max.}$ 499 mμ (*D* 0·540; representing 600 μg. of urobilin).

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