The *meso*-Reactivity of Porphyrins and Related Compounds. Part VI.[1] Oxidative Cleavage of the Haem System. The Four Isomeric Biliverdins of the IX Series[2]

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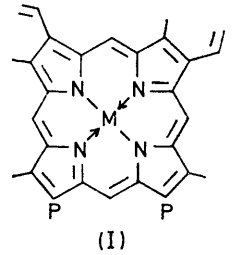
The oxidative cleavage of haemin in aqueous pyridine under various conditions (ascorbic acid–oxygen; hydrazine–oxygen; and ascorbic acid–hydrogen peroxide) gives four isomeric biliverdins of the IX series which have been isolated (from a much improved ascorbic acid–oxygen reaction) as their crystalline dimethyl esters. The properties of the four isomers are described. On the basis of mass and n.m.r. spectra, structures are assigned to all four isomers for the first time. A key observation in the n.m.r. spectra of these compounds is the chemical shift of the heteroaryl methyl group. The implications of these results are discussed.

THE oxidative cleavage (for example, with O_2–ascorbic acid, followed by hydrolysis) of bispyridine protohaemochrome (I; M = py_2Fe^{II}) *in vitro* has been extensively investigated because of its possible relevance to haem catabolism.[1,3-5] Although it is generally agreed that the model process leads eventually to the bilatriene system, the question of the positional specificity of the reaction has not been satisfactorily resolved. This is important in the role of this system as a model for the *in vivo* reaction since if the substituents of the haem system (I) are capable of directing reaction to C-5 (the α-position) then the reaction would closely resemble the catabolic process in that biliverdin IXα (II) would result. If, on the other hand, the vinyl and propionic acid side-chains exert little or no directing effect, a mixture of all four verdin isomers (II)—(V) would be expected, and, by implication, some sort of direction, presumably enzymatic, would be required *in vivo* to cause the reaction to occur specifically at C-5.

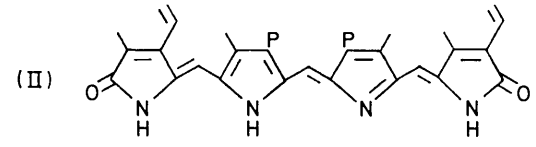
The first observations concerning the specificity of the *meso*-cleavage of the system (I) were made by Lemberg who concluded (ref. 3, p. 460) that the cleavage (O_2–ascorbic acid) gave the α-isomer (II); no other isomer was detected. However, in 1962 Gray and his colleagues[6] reported that a mixture of biliverdins IX was formed during coupled oxidation with O_2–hydrazine. Recently Rüdiger[7] and Ó Carra[8] have reported that all four isomers are formed, whereas Nichol and Morell[9] have concluded that the product is the β- (or δ-) isomer



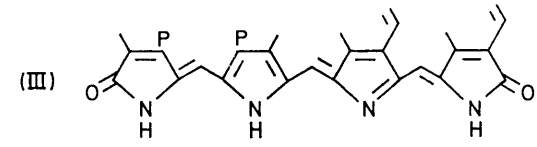
(I)

P = $CH_2{\cdot}CH_2{\cdot}CO_2H$

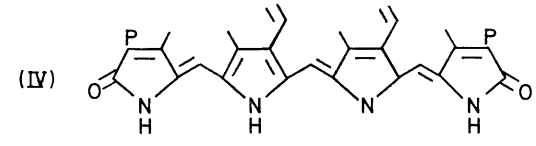
The suffix a refers to dimethyl esters throughout



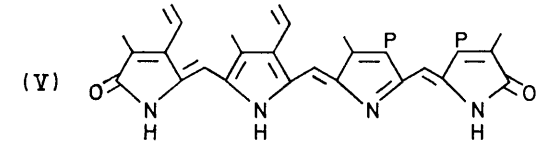
(II) Biliverdin (component 2) IXα



(III) Biliverdin (component 1) IXβ



(IV) Biliverdin (component 3) IXγ



(V) Biliverdin (component 4) IXδ

[1] Part V, R. Bonnett and M. J. Dimsdale, *J.C.S. Perkin I*, 1972, 2540.

[2] Preliminary communication, R. Bonnett and A. F. McDonagh, *Chem. Comm.*, 1970, 237.

[3] R. Lemberg and J. W. Legge, 'Hematin Compounds and Bile Pigments,' Interscience, New York, 1949.

[4] R. Lemberg, *Rev. Pure Appl. Chem.*, 1956, **6**, 1.

[5] C. Ó hEocha in 'Porphyrins and Related Compounds,' Biochemical Society Symposium No. 28, ed. T. W. Goodwin, Academic Press, London, 1968, p. 91.

[6] Z. Petryka, D. C. Nicholson, and C. H. Gray, *Nature*, 1962, **194**, 1047.

[7] W. Rüdiger, in ref. 5, p. 121; *Z. physiol. Chem.*, 1969, **350**, 1291.

[8] E. Colleran and P. Ó Carra, *Biochem. J.*, 1969, **115**, 13P.

[9] A. W. Nichol and D. B. Morell, *Biochim. Biophys. Acta*, 1969, **184**, 173.

(III) [or (V)]. In no instance have all four verdins been isolated in crystalline form and identified.

Preliminary experiments were directed at improving the yield of verdohaemochrome by use of Lemberg's system (haemin–aqueous pyridine–O_2–ascorbic acid) with the following results: (i) The use of veronal buffers as described by Nakajima [10] and by Levin [11] was found to lead to difficulties in purification, the veronal tending to contaminate the product. It was preferable, indeed, to rely on the buffering properties of the aqueous pyridine–ascorbic acid solution itself, which appeared to change very little from neutrality (pH *ca.* 7·4—7·7) during the (short) reaction time employed. (ii) In small scale preparations (25 mg) it was found that the yield of verdohaemochrome was very sensitive to the treatment of the sample before the addition of ascorbic acid, best results being observed when the solution of haemin (I; M = $ClFe^{III}$) in aqueous pyridine at 37° was saturated

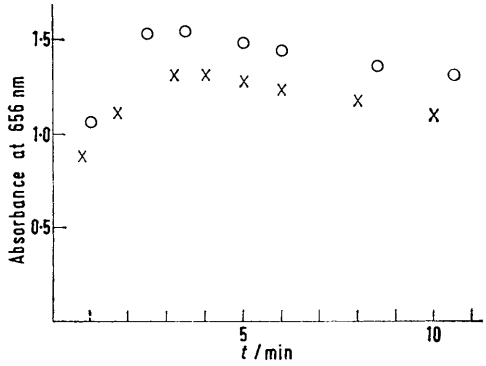


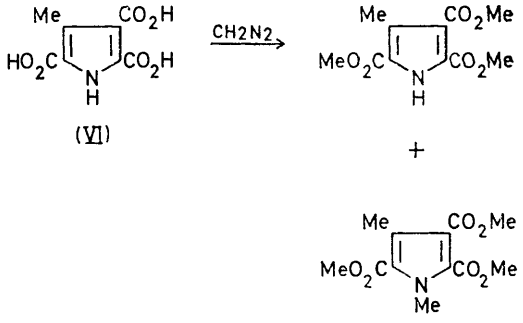
FIGURE 1 Formation of verdohaemochrome in coupled oxidation of haemin with ascorbic acid (for conditions see Experimental section); ○ samples examined directly; × samples examined after quenching with acetic acid

with oxygen before the reducing agent was added. (iii) The duration of the reaction was found to be a significant factor. Lemberg's early results [12] had indicated that short reaction periods could be used, with obvious advantages, but recent workers have tended to employ longer reaction periods (*e.g.* ref. 10, 40 min; ref. 9, 3 h). In our experiment the maximum yield of verdohaemochrome was produced after 3 min (Figure 1).

In the procedure which eventually emerged, haemin in dilute solution (water–pyridine, 3 : 1) at 37° and saturated with oxygen is treated with a large excess of ascorbic acid with vigorous agitation for 3 min. (Under these conditions haemin is unaffected in the absence of ascorbic acid.) The reaction is quenched, and the crude verdohaemochrome is isolated without delay. Cleavage and esterification (KOH–MeOH, then BF_3–MeOH), followed by a brief chromatographic treatment to remove polar material, then gives the mixed biliverdin methyl esters in 42% yield. This is a marked improvement on earlier procedures, where yields of about 5% or less have been commonly recorded.[6,9,12]

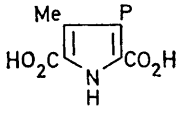
[10] H. Nakajima, *J. Biol. Chem.*, 1963, **238**, 3797.
[11] E. Y. Levin, *Biochemistry*, 1966, **5**, 2845.
[12] R. Lemberg, *Biochem. J.*, 1935, **29**, 1322; R. Lemberg, B. Cortis-Jones, and M. Norrie, *ibid.*, 1938, **32**, 149.

For the analysis of verdin samples we initially considered the Nicolaus oxidation procedure (permanganate oxidation of polypyrroles to give pyrrolepolycarboxylic acids) [13] but soon abandoned this approach when it became apparent that the oxidation products were not stable under the reaction conditions, thus making



SCHEME 1

quantitative work unreliable. Another difficulty arose when treatment of the tricarboxylic acid (VI) with diazomethane (to give esters for g.l.c. and t.l.c. analysis) not unexpectedly gave mixtures in which some *N*-methylated products were present (Scheme 1). This type of reaction has also been reported recently by Swan and Waggott.[14] Although an oxidation method has, in conjunction with t.l.c., been developed by Rüdiger [7] into a very useful approach, uncertainty is inherent when an oxidation procedure is applied directly to mixtures of verdins. Thus the same oxidation result



(VII)

[a mixture of the acids (VI) and (VII) obtained on oxidising a verdin sample] was interpreted by Petryka, Nicholson, and Gray [6] as indicating an unspecified mixture of verdins [(II), (III), (IV), and (V)], and by Nichol and Morell [9] in terms of a single isomer [β- or δ-, (III) or (V)]. Because of these difficulties it was decided to develop a separation procedure to prepare the individual isomeric esters in small quantity.

The mixed ester preparation was found to separate into four main components on analytical t.l.c. under carefully controlled conditions (silica gel; 29°; 3% acetone–chloroform; see Experimental section). A small portion of the mixture was separated under analytical conditions to give the individual components, obtained crystalline from chloroform–light petroleum containing a trace of methanol, in milligram amounts. The bulk of the material was separated on thick layers of silica, but here the two more mobile compounds (β- and α-, as it emerged) could not be separated cleanly, so that

[13] C. H. Gray, D. C. Nicholson, and R. A. Nicolaus, *Nature*, 1958, **181**, 183; R. A. Nicolaus, *Rassegna di Medicina Sperimentale, Suppl.* 2, 1960, **7**, 1.
[14] G. A. Swan and A. Waggott, *J. Chem. Soc.* (*C*), 1970, 285.

three fractions ($\beta + \alpha$, γ, δ) were obtained. Other workers have independently developed t.l.c. systems which will separate isomeric biliverdin esters.[7,15,16] A two-dimensional t.l.c. system is shown diagrammatically

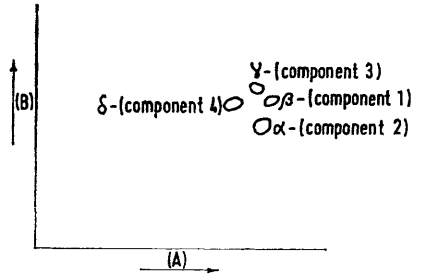
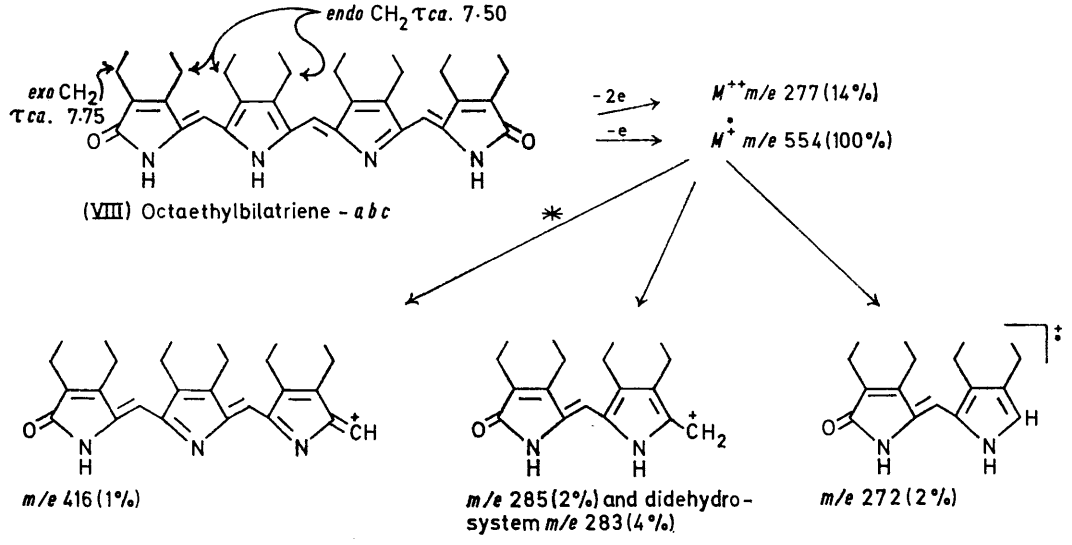


FIGURE 2 Two-dimensional t.l.c. of the four verdin esters on Merck Silica Gel G (0·25 mm; ambient temperature in the dark); (A) first development (90 min) with 3% acetone in reagent grade chloroform; (B) second development (60 min) with petroleum–acetone–propionic acid (12 : 3 : 1). We thank Mrs. P. Bray for these observations

in Figure 2; it is convenient to refer to the components in the following sections in the order of the mobilities they possess in the 3% acetone–chloroform system.

ester was obtained, and was identical (mixed m.p., mixed t.l.c., electronic spectrum, mass spectrum) with the second most mobile component (labelled 2 in Figure 2) in the acetone–chloroform system.

Under electron impact biliverdins are known to fragment about the central methine bridge.[18] Thus octaethylbilatriene-*abc* (VIII), which we have found to be a most useful model in this series,[1] gives fragments as rationalised in Scheme 2. (A small amount of cleavage at the alternative methine bridge is confirmed by the appearance of a metastable peak at *m/e* 312·8.) Biliverdin IXα dimethyl ester (component 2) and component 3 showed important fragment ions at *m/e* 313, 311, and 300 (the latter being the base peak for component 3). These fragments are formulated as (a) and (b) or the positional isomers of these structures. Hence component 3 is biliverdin IXγ dimethyl ester (IVa). The mass spectra of components 1 and 4 showed significant fragments at *m/e* 360, 253, and 251 (relatively weak in 4) attributed to the ions (c) and (d). These fragment ions agreed with, but did not distinguish between, structures (IIIa) and (Va).

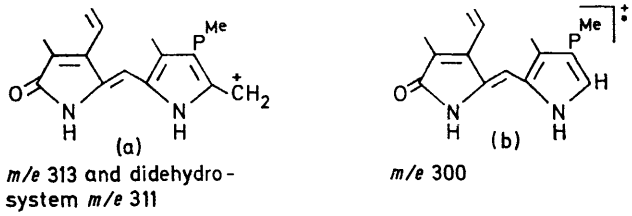


SCHEME 2 Fragmentation of octaethylbilatriene-*abc* under electron impact (probe at 243°)

The mass spectra of the four crystalline verdin esters showed molecular ions at *m/e* 610, and accurate mass measurement in each case agreed with the molecular formula $C_{35}H_{38}N_4O_6$. The problem of the assignment of structures to the individual components was tackled as follows.

Biliverdin IXα dimethyl ester was available from the dehydrogenation of natural bilirubin. This reaction was found to be much more complex than the literature suggests, since a randomisation of the rubin occurs in which isomers of the III and XIII series are produced.[17] However, a pure sample of biliverdin IXα dimethyl

This distinction was made on the basis of the n.m.r. spectra of the four isomeric esters. Again octaethyl-



bilatriene-*abc* (VIII) played an important role as a model system, since it had been noted that the alkyl groups a

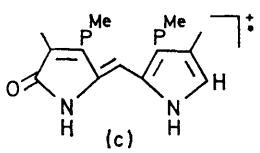
[15] *Cf.* also M. S. Stoll and C. H. Gray, *Biochem. J.*, 1970, **117**, 271.

[16] P. Ó Carra and E. Colleran, *J. Chromatography*, 1970, **50**, 458.

[17] R. Bonnett and A. F. McDonagh, *Chem. Comm.*, 1970, 238; *cf.* A. F. McDonagh and F. Assisi, *J.C.S. Chem. Comm.*, 1972, 117.

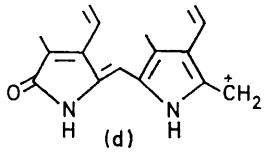
[18] A. H. Jackson and G. W. Kenner, in ref. 5, p. 3.

the ' outside ' positions (which we have termed *exo*) are shielded with respect to the alkyl groups at other (*endo*) β-positions. The effect is indicated for the methylene



(c)

m/e 360



(d)

or positional isomer *m/e* 253 and didehydro-system *m/e* 251

groups in structure (VIII) (in Scheme 2). The difference in chemical shift presumably arises because the *endo*-β-positions are flanked, and deshielded, by two aromatic rings, whereas the *exo*-β-positions are markedly affected by only one (even for a macrocyclic conformation). In the n.m.r. spectra of the four isomeric esters (IIa)—(Va) the *C*-methyl signals fall into two ranges: τ *ca.* 8·1—8·3 (*exo*) and τ *ca.* 7·7—7·9 (*endo*). (Concentration effects have been observed in the porphyrin series,[19] but studies with biliverdin IXα dimethyl ester showed that they are not significant here in the relevant concentration range; see Experimental section.) The n.m.r. spectrum of component 1 showed a signal for one *exo*-*C*-methyl at τ 8·26 and component 1 was therefore the β-isomer (IIIa); this left the δ-structure (Va) for component 4, confirmed by the n.m.r. observations (signals for two *C*-methyls at τ 8·10 and 8·17). As shown in Table 1, in which is abstracted the spectroscopic data relevant to the structural elucidation, the n.m.r. and mass spectrometric data reinforce each other, and allow unique structural assignments to be made to the four isolated isomers for the first time.

Since these results were communicated Ô Carra and Colleran [16] have reported an alternative procedure for assigning individual structures to the β/δ pair of isomers. The biliverdins were reduced to mesobiliverdins, and these materials were compared (mixed t.l.c. only) with a synthetic sample of mesobiliverdin IXβ dimethyl ester; this identified the β-isomer, and the δ-structure was assigned by elimination. While this analysis appears to be less conclusive than the procedure adopted here, an exchange of samples between Ô Carra's laboratory and our own has revealed that the two groups agree on the structures of the individual components.

A comment on the results of Nichol and Morell [9] is also desirable since, during the course of our studies, they reported that the coupled oxidation (O_2–ascorbic acid) of pyridine protohaemochrome gave not four isomers but a single isomer which, as the dimethyl ester, was obtained crystalline and identified (mass spectrometry, permanganate oxidation) as the β- or δ-isomer [(IIIa) or (Va)]. However the n.m.r. spectrum reported [9] (for a preparation in which hydrazine was used as the reductant) corresponded to that of the substance which we regard as the γ-isomer. Recently Dr. Nichol has provided us with a sample of his preparation of the β(δ)-diester. Comparisons have shown that this is essentially biliverdin IXβ dimethyl ester: the n.m.r. spectrum of this sample was recorded and corresponded not to that of the γ-isomer (*i.e.* Figure 2B of ref. 9; and see later) but to that of the β-isomer (see later). Thus it appears that Nichol and Morell had obtained purified samples of the β- and the γ-isomers. While lengthy reaction times (and consequent low yields) may account to some degree for variation in isomer distribution, the most likely explanation for this result is chance crystallisation of a single isomer. Other examples of this behaviour have been noted in this series: thus Lemberg [12] records that from coupled oxidation of mesohaemin he obtained two crystalline mesobiliverdin dimethyl esters

TABLE 1

Properties of the isomeric biliverdin IX dimethyl esters [a]

Component	Structure	M.p. (°C) [b]	$\lambda_{max.}$/nm ($CHCl_3$)	*R* [c]	M^+	Some bipyrrolic fragment ions (*m/e*)	N.m.r.[d] (ArMe) *endo*	N.m.r.[d] (ArMe) *exo*
1	(IIIa)β	212—214	381, 648—654	3·29	610·280[e,f]	360(3) 253(4) 251(9)	7·71 7·80 7·86	8·26
2	(IIa)α	205—207	379, 652—660	3·30	610·280 [e]	313(3) 311(4) 300(3)	7·86 7·91 7·94	8·19
3	(IVa)γ	205—207	376, 639	3·13	610·279	313(13) 311(13) 300 [e]	7·77 7·87 7·90(6H)	
4	(Va)δ	172—174	379, 651—657	2·86	610·281 [g]	373(4) 360 [e] 253(9) 251(4) 240(23)	7·82 7·84	8·10 8·17

[a] Presented in order of decreasing mobility on the t.l.c. plate. [b] Kofler–Reichert hot stage. [c] Ratio of extinction of the two peaks $\varepsilon_{ca.\ 380}/\varepsilon_{ca.\ 650}$). [d] Insufficient amounts of pure α- and β-isomers were available for this determination; the quoted (τ) values refer to the mixture of these obtained on ' large-scale ' preparative t.l.c. The signals due to the β-isomer were ascertained by subtracting those found in authentic biliverdin IXα dimethyl ester from the spectrum of the mixture. [e] Base peak. [f] Calc. for $C_{35}H_{38}N_4O_6$, M^+, 610·279. [g] A peak at *m/e* 612 was also observed (see text).

[19] R. J. Abraham, P. A. Burbidge, A. H. Jackson, and G. W. Kenner, *Proc. Chem. Soc.*, 1963, 134.

(m.p. 179—180 and 218—219°), which were regarded [3] as isomers; and Ó Carra has observed [20] the separation of a single isomer (thought to be deuterobiliverdin IXβ dimethyl ester) from a deuterohaemin cleavage.

Properties of the Individual Crystalline Isomers.— Some of the physical properties of the four isomeric esters are presented in Table 1; other details are collected in the Experimental section. A brief commentary on notable spectroscopic features is given here.

The i.r. spectra were recorded (KBr discs) for each isomer except β (insufficient pure material). Ester carbonyl stretching appeared at *ca.* 1740 cm^{-1}, with the γ-isomer showing a second, weaker band, presumably indicating hydrogen bonding, at 1731 cm^{-1}. Lactam carbonyl absorption was observed at *ca.* 1700 and 1675 cm^{-1}.

The electronic spectra (chloroform) showed small differences attributed to the slight changes in electron delocalisation with the varying positions of the vinyl groups. Of all the isomers, the γ-isomer, with vinyl groups on rings B and C, had maxima at shortest wavelengths. Nevertheless these differences are insufficient in themselves to form a reliable basis for distinguishing between the isomers, especially since the low energy absorption is so broad that its maximum is difficult to locate with precision.

The n.m.r. spectra (deuteriochloroform) are presented in Figure 3. Since, until the arrival of the Nichol and Morell sample, insufficient pure biliverdin IXβ dimethyl ester was available for n.m.r. study, the chemical shifts for this isomer were ascertained by subtracting those found for authentic biliverdin IXα dimethyl ester from the spectrum of the αβ-mixture obtained from the thick-layer preparation (see Experimental section).

The methylene groups of the propionic ester function appear as expected as two multiplets (broad triplets) in the region τ *ca.* 7—7·5 except for the γ-isomer. Here a singlet is observed at τ 7·49. This is the only isomer with propionic ester functions at the *exo*-positions: it is thought that the methylene protons adjacent to the ring are less deshielded than in the other isomers and, by chance, their chemical shift is virtually identical with that of the neighbouring methylene protons, and no coupling is detected. The methoxy-groups of the propionic ester functions appear as singlets in the spectra of the α- and γ-isomers, but two signals are resolved in the case of the β- and δ-isomers. The *meso*-protons appear as singlets at τ *ca.* 4·05 for the *a* and *c* bridges, and at τ *ca.* 3·2 for the *b* bridge. The vinyl groups give rise to multiplets at τ *ca.* 3·5 (–CH=) and *ca.* 4·5 ($=CH_2$).

In the mass spectra (see Experimental section) the molecular ion is the base peak for the α- and β-isomers, whereas for the γ- and δ-isomers a bipyrrolic fragment is the base peak (300 and 360 respectively): the molecular ion remains prominent for the γ-isomer (81%) but is much less so for the δ-isomer (6%). However too much significance should not be attached to such variations since in this series relative abundance is particularly sensitive to operating conditions (see, for

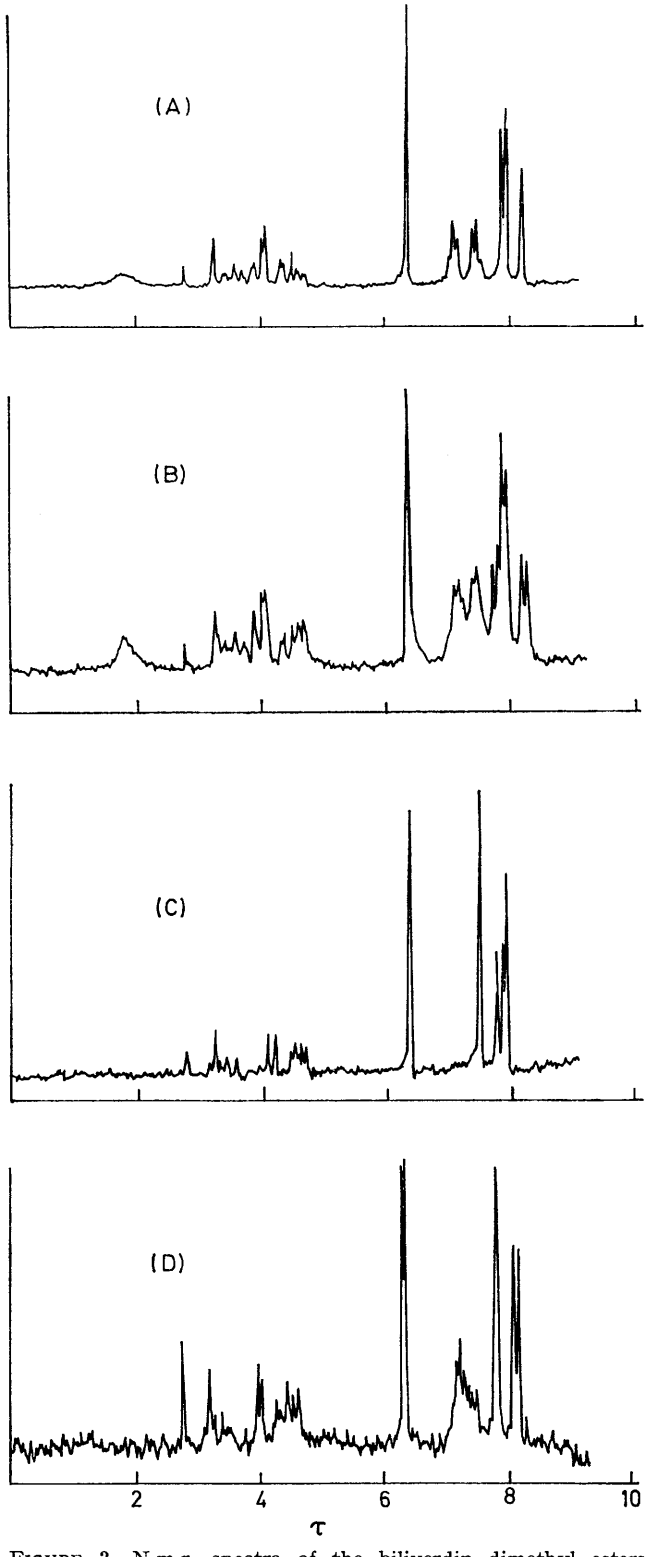


FIGURE 3 N.m.r. spectra of the biliverdin dimethyl esters ($CDCl_3$; 100 MHz); (A) biliverdin IXa dimethyl ester from dehydrogenation of bilirubin; [17] (B) mixture of IXα and IXβ isomeric esters (components 1 and 2); (C) biliverdin IXγ dimethyl ester (component 3); (D) biliverdin IXδ dimethyl ester (component 4)

[20] P. Ó Carra, personal communication.

example, the mass spectra of synthetic mesobiliverdin IXβ dimethyl ester in refs. 15 and 18). The δ-isomer showed, beside the molecular ion at *m/e* 610, a peak at *m/e* 612. Such '$M + 2$' peaks have been observed on other occasions in the tetrapyrrole series [21] and have been attributed to hydrogen-transfer processes occurring on the probe. The absence of a triplet in the high field regions of the n.m.r. spectrum shows that the $M + 2$ peak cannot be attributed to appreciable contamination with a dihydrobiliverdin dimethyl ester.

Besides cleavage to bipyrrolic fragments (Table 1) other expected features such as side-chain cleavage (M — OMe, M — CO_2Me, M — CH_2CO_2Me), doubly charged molecular ions, and metastable ions (see Experimental section) were observed although all these features were more prominent in the spectra of the α- and β-isomers, which had the molecular ion as the base peak, than in those of the γ- and δ-esters, which had not.

Conclusion.—Cleavage of bispyridine protohaemochrome with O_2–ascorbic acid, followed by hydrolysis, gives a mixture of the four possible verdin isomers (II)—(V). Spectroscopic estimation [2] of the relative abundance of the isomers (as esters) gives α, 32%; β, 22%; γ, 18%; and δ, 28%. Although the α-cleavage product is the marginally the most important, it does not represent a dominant pathway. Clearly the substituents exert little directing effect in this reaction, and although, in our view, the coupled oxidation and the intermediates therein [1,4] provide a plausible interpretation of the fundamental chemistry of haem catabolism, the manner in which positional specificity is achieved *in vivo* is still far from clear. Positional specificity may be achieved at an enzyme surface, but this is not easily envisaged if the globin remains attached to the haem system, since, of the four *meso*-positions, the α-position (C-5) is the most deeply buried in the globin system. It is interesting to note that the possibility that the globin itself directs the reaction has recently received some experimental support.[22] We have found that coupled oxidation of freshly crystallised human oxyhaemoglobin with ascorbic acid–air gives biliverdin IXα dimethyl ester as the main product, which has been isolated in crystalline form: traces of the β- and δ-isomers were also detected by t.l.c. Similar observations have been reported by Nichol and Morell [9] and by Ó Carra and Colleran.[22] While these results clearly suggest a directing role for the globin in the cleavage process, they need to be treated with circumspection unless the verdin is isolated in good yield.

With the solution of the biliverdin isomer problem, and the development of a rapid t.l.c. method for separating the four isomers as their esters, a useful analytical method is at hand which, it is thought, will find several biochemical applications. It has considerable potential, for example, in the search for the 'wrong' biliverdin isomers in nature (*cf.* biliverdin IXγ, reported [23] as the tegumental pigment of caterpillars of *Pieris brassicae*) and offers a subtle porphyrin degradation which may prove valuable in studies on the post-porphobilinogen stages of porphyrin biosynthesis.[24]

[21] H. Budzikiewicz and S. E. Drewes, *Annalen*, 1968, **716**, 222.
[22] P. Ó Carra and E. Colleran, *FEBS Letters*, 1969, **5**, 295.

EXPERIMENTAL

The following spectroscopic facilities were employed: electronic spectra, Unicam SP 800B, calibrated with holmium glass and reported as λ/nm (ε) for maxima (unless otherwise otherwise stated); i.r. spectra, Perkin-Elmer 225, KBr discs, reported as $\nu_{max.}/cm^{-1}$; n.m.r. spectra, Varian HA100, in $CDCl_3$ with tetramethylsilane as internal reference, reported as τ values; mass spectra, A.E.I. MS902, ionising voltage 70 eV, direct insertion, probe temperature indicated, calibrated with heptacosafluorotributylamine and reported as *m/e* values for significant ions with *m/e* >240 with relative abundances in parentheses (%).

Petroleum refers to that fraction of b.p. 60—80°. Chloroform refers to reagent grade (May & Baker Ltd.) containing 1% ethanol as stabiliser. Solvent compositions are given by volume.

T.l.c. plates were prepared with Merck Silica Gel G, following the manufacturer's instructions, and using glass-distilled water to prepare the slurry.

For analytical work, plates (0·25 mm thick) were developed for about 90 min at 29° with 3% acetone–chloroform in unlined closed tanks kept in a draught-free enclosure in the dark. The individual verdin esters reported here were homogenous in this system, and also in carbon tetrachloride–methyl acetate (3 : 1) development. A two-dimensional separation was also used analytically (Figure 2). For preparative work the plates were irrigated with methanol overnight, and reactivated before use. Pigments were applied as dilute solutions (8—10 mg ml^{-1}) in chloroform in subdued light, and the chloroform was removed with a stream of nitrogen. After development, individual pigments were extracted with acetone. The extract was filtered, the solvent was removed (reduced pressure), the residue was dissolved in chloroform, and the solution was again filtered (Celite). Concentration of the filtrate and careful addition of petroleum generally gave a pure crystalline verdin.

Verdinoid free acids were methylated by refluxing a solution or suspension of the pigment in anhydrous methanol (nitrogen purged) with methanolic 14% boron trifluoride (*ca.* 0·5 vol) for 15 min under nitrogen. After being kept overnight at 4° in the dark, the solution was diluted with water (*ca.* 3 vol.) and extracted with chloroform to remove the pigment, the organic extract being twice washed with water. The resulting chloroform solution was filtered through chloroform-moistened filter paper and evaporated under reduced pressure.

Verdohaemochrome Formation from Haemin–Oxygen–Ascorbic Acid. Effect of Time.—Haemin was treated with oxygen–ascorbic acid exactly as described in the next experiment except that the incubation was continued for 11 min. During the reaction absorption due to verdohaemochrome ($\lambda_{max.}$ 498, 534, and 656 in this solution) was measured both directly and on samples (5 ml) of solution which were mixed with acetic acid (2 ml) immediately after withdrawal (see Figure 1).

Oxidation of Haemin with Oxygen–Ascorbic Acid.—The

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[24] A. R. Battersby, Proc. 23rd Internat. Congr. Pure Appl. Chem., Butterworth, London, 1971, vol. 5, p. 1.

entire experiment was carried out in subdued light. Oxygen was bubbled briskly through a solution of haemin (25 mg) in aqueous pyridine (3 : 1; 400 ml) at 37° for 20 min. The oxygen inlet tube was then withdrawn to just above the surface of the solution, rapid stirring was started, and a solution of ascorbic acid (300 mg) in water (5 ml) was added from a pipette. Three minutes (stop-clock) after completion of the addition the solution (pH *ca.* 7·7) was poured into ice-cold chloroform (100 ml) under nitrogen. The mixture was shaken and the layers were separated without delay. The aqueous layer was washed with chloroform (30 ml), the chloroform extracts being washed at once with water (100 ml; N_2 flush), and dried briefly (Na_2SO_4; 5—10 min) under nitrogen. The filtered solution was concentrated under reduced pressure (bath 43°) to a *moist* green residue.

The combined products from four such experiments were dissolved in chloroform (5 ml), and the solution was diluted with petroleum (30 ml). The precipitate was collected by centrifugation, washed twice with petroleum at the centrifuge, and dried under reduced pressure to give crude amorphous verdohaemochrome, λ (1% pyridine in $CHCl_3$; extinction ratios) 396 (4·1), 502 (0·7), 535 (1·0), and 663 (2·2).

The crude verdohaemochrome in methanol (25 ml; N_2 flush) was treated with methanolic potassium hydroxide (2N; 2 ml) in the dark. Boron trifluoride in methanol (14%; 25 ml) was added after 1 min, and the solution was refluxed under nitrogen for 15 min and then kept at room temperature overnight in the dark before being worked up by the general method (see before) to give the crude esters (69 mg). This material was chromatographed on a short column of alumina (activity V made up in benzene; material applied in chloroform–benzene, 1 : 9) and the blue-green band was eluted with chloroform–benzene (9 : 1) without delay to give 40 mg (42%) of purified mixed biliverdin IX dimethyl esters. [Alternatively the dried chloroform solution of the crude esters was concentrated to *ca.* 20 ml, and filtered onto a pad of Silica Gel G (Merck; 2 cm diam., 0·5 cm thick): this adsorbed the pigment, the major verdin components of which were eluted without delay by acetone–chloroform (1 : 4).] A small portion of this material was separated by analytical t.l.c. (to give the α-, β-, γ-, and δ-isomers as individuals); the bulk of the material was separated into three fractions (α + β, γ, δ) by preparative t.l.c.

Small-scale Separation.—A small portion of the biliverdin dimethyl ester mixture was separated into four components by t.l.c. on fourteen analytical plates (Silica Gel G, 20 × 20 cm × 0·25 mm; 3% acetone–chloroform; 29°; 1·5 h) and the products were crystallised from chloroform–petroleum containing a trace of methanol. Occasionally when two bands, or parts of two bands, did not separate cleanly they were discarded. There were obtained, in order of decreasing mobility on the plate:

(i) *Biliverdin IXβ dimethyl ester*, microcrystalline (platelets?), m.p. 212—214° (decomp.), *m/e* (218°) 610 (100), 595 (5), 579 (6), 551 (3), 537 (6), 523 (3), 521 (3), 388 (3), 360 (3), 305 (M^{2+}, 4), 268 (6), 266 (4), 253 (4), 252 (9), and 251 (9), *m** 580·5 (610 ⟶ 595 = 580·4) and 473 (610 ⟶ 537 = 472·9).

(ii) *Biliverdin IXα dimethyl ester*, blades, m.p. 205—207° (mixed m.p. 206—208° with an authentic sample [17]), *m/e* (197°) 610 (100), 579 (5), 551 (3), 537 (4), 523 (3), 463 (4), 450 (3), 313 (3), 311 (4), 305 (M^{2+}, 10), and 300 (3), *m** 580·5, 549 (610 ⟶ 579 = 549·6), 498 (610 ⟶ 551 = 498·1), 493, and 449 (610 ⟶ 523 = 448·4).

(iii) *Biliverdin IXγ dimethyl ester*, needles, m.p. 205—207° (mixed m.p. with α-isomer, 192—202°), *m/e* (208°) 610 (81), 595 (15), 326 (21), 313 (13), 311 (13), 300 (100), 285 (22), 269 (20), 261 (28), 241 (23), and 240 (36).

(iv) *Biliverdin IXδ dimethyl ester*, rosettes of blades, m.p. 172—174°, *m/e* (260°) 612 (6), 610 (6), 491 (5), 374 (27), 360 (100), 342 (6), 329 (14), 328 (18), 314 (7), 302 (7), 301 (19), 300 (15), 299 (9), 298 (6), 288 (9), 287 (41), 286 (9), 285 (16), 255 (25), 254 (9), 253 (9), 242 (15), 241 (18), and 240 (23).

Large-scale Separation.—Large-scale preparative t.l.c. (Silica Gel H, 1·5 mm; 5% acetone–chloroform; 7 h) of the bulk of the product gave three bands. The verdin esters were extracted (11, 5, and 6 mg, from upper, middle, and lower bands, respectively) and crystallised to give, in order of decreasing mobility:

(i) A mixture (8·5 mg) of biliverdins IXβ and IXα dimethyl esters, m.p. 196—204°, τ ($CDCl_3$; Me signals only) 6·36, 6·37 (scale expansion), 7·71, 7·80, 7·86, 7·91, 7·94, 8·19, 8·26.

(ii) Biliverdin IXγ dimethyl ester (2·5 mg), m.p. 207—209°, λ ($CHCl_3$) 376 (54,400) and 639 (18,100), ν (KBr) 1740, 1731, 1698, and 1687, τ ($CDCl_3$) 3·24 (s, *b* bridge), 3·32—3·57 (m, C*H*=CH_2), 4·08 and 4·20 (singlets, *a* and *c* bridges), 4·46—4·71 (m, −CH=C*H*$_2$), 6·38 (s, OMe), 7·49 (s, C*H*$_2$–C*H*$_2$), and 7·77, 7·87, 7·90, and 7·90 (singlets, *endo*-Ar*Me*).

(iii) Biliverdin IXδ dimethyl ester (3·1 mg), m.p. 172—174°, λ ($CHCl_3$) 379 (43,600) and 651—657 (15,000), ν (KBr) 1738, 1701, and 1689, τ ($CDCl_3$) 3·18 (s, *b* bridge), 3·10—3·54 (m, −C*H*=CH_2), 3·96 and 4·02 (singlets, *a* and *c* bridges), 4·28—4·61 (m, CH=C*H*$_2$), 6·32 and 6·36 (singlets, OMe), 7·06—7·54 (m, C*H*$_2$–C*H*$_2$), 7·82 and 7·84 (singlets, *endo*-Ar*Me*), and 8·10 and 8·17 (singlets, *exo*-Ar*Me*).

Oxidation of Haemin with Oxygen–Hydrazine.[6]—Oxygen was passed briskly through a solution of haemin (100 mg) in aqueous pyridine (5 : 2; 490 ml) at 52°. Hydrazine sulphate (260 mg) in 0·5N-NaOH (6·6 ml) was added, and the solution was kept at 52° for 10 min. The solution was kept in an ice-bath for 25 min and then extracted with chloroform (1 × 100; 3 × 50 ml). The organic layer was dried (Na_2SO_4), filtered, and concentrated (40°; reduced pressure) to near dryness. The green residue was converted into biliverdin esters as before. The crude product (67 mg) in a small volume of chloroform was filtered through a pad of Silica Gel G, the verdin esters being washed through without delay with chloroform to yield, on evaporation, a green solid (36 mg, 38%) shown by analytical t.l.c. to consist largely of the four biliverdin IX dimethyl esters already described.

Similarly treatment of haemin in pyridine–water with ascorbic acid and hydrogen peroxide,[25] followed by oxygenation and cleavage, gave a mixture of the four verdin esters (t.l.c.).

Coupled Oxidation of Human Haemoglobin.[26]—Moist crystalline human oxyhaemoglobin (23 g; prepared by the method of Hinson and McMeekin [27] from freshly drawn

[25] R. Lemberg, B. Cortis-Jones, and M. Norrie, *Biochem. J.*, 1938, **32**, 171.

[26] R. Lemberg, W. H. Lockwood, and J. W. Legge, *Biochem. J.*, 1941, **35**, 363.

[27] J. A. Hinson and T. L. McMeekin, *Biochem. Biophys. Res. Comm.*, 1969, **35**, 94.

blood) was dissolved in 0·1M-phosphate buffer (600 ml; pH 8·5; 0·1M-KH_2PO_4 + 0·1N-NaOH). The solution was divided evenly between twelve 250 ml Ehrlenmeyer flasks immersed in a bath at 37°, and each was treated with a solution of ascorbic acid (50 mg) adjusted to pH 8·5 in 0·1M-phosphate buffer (5 ml). The flasks were gently swirled a few times, and then kept unstoppered at 37° for 2·25 h. The solutions were then poured into acetic acid (1200 ml) and the verdins were isolated by extraction into ether, followed by extraction of the ethereal solution with aqueous sodium acetate, water, and (exhaustively) 5% (w/v) hydrochloric acid as described by Lemberg.[26] The combined acid extracts were concentrated (rotary evaporator; 38°) and the residue was methylated and worked up in the usual way. T.l.c. at this stage showed one major and several minor components, together with polar material. The crude product in chloroform was adsorbed onto a pad of Silica Gel G, and verdin esters were eluted with 20% acetone–chloroform (10 ml). Preparative t.l.c. (Silica Gel G; 1 mm; 5% acetone–chloroform) gave one main and several minor bands (see later). The main band yielded 1·8 mg (2% yield, estimated spectroscopically) of biliverdin IXα dimethyl ester, which was crystallised from chloroform–petroleum to give green blades, m.p. 208—209°. The identity of this material was confirmed by comparison (t.l.c., i.r. and mass spectra, and mixed m.p. 207—208°) with an authentic sample. The examination of the mother liquors indicated a very small proportion of biliverdin IXβ dimethyl ester.

The minor bands from the preparative t.l.c. separation were examined by analytical t.l.c. One was identified as biliverdin IXδ dimethyl ester, formed in only trace amount; biliverdin IXγ was not detected.

N.m.r. Spectrum of Biliverdin IXα Dimethyl Ester in Chloroform. Dilution Studies.—The n.m.r. spectrum of biliverdin IXα dimethyl ester in $CDCl_3$ was measured over the accessible concentration range. Chemical shifts (τ) are given in Table 2.

Reaction of 3-Methylpyrrole-2,4,5-tricarboxylic Acid with Diazomethane.—3-Methylpyrrole-2,4,5-tricarboxylic acid [28] (102 mg) as a slurry stirred in ether was treated at 0° with an excess of ethereal diazomethane. After 1 h the excess of diazomethane was destroyed (HOAc); the solution was washed (water, aqueous sodium hydrogen carbonate), dried ($MgSO_4$), and taken to dryness. Preparative t.l.c. (Silica Gel HF_{254}, 0·5 mm; 20% acetone–petroleum) gave two components which were extracted with methanol. The lower band yielded trimethyl 3-methylpyrrole-2,4,5-tricarboxylate (42 mg, 34%), m.p. (sealed capillary) 143—146° (lit.,[28] 142—143°); the upper band gave *trimethyl 1,3-dimethylpyrrole-2,4,5-tricarboxylate* (56 mg, 43%), which was obtained (48 mg) as prisms, m.p. 59—62° (sealed capillary) after sublimation (Found: C, 53·8; H, 5·3; N, 5·05%; M^+, 269. $C_{12}H_{15}NO_6$ requires C, 53·55; H, 5·6; N, 5·2%; *M*, 269), λ (MeOH) 224 (20,600) and 269 (11,800), τ ($CDCl_3$) 6·03 (s), 6·10 (s), 6·11 (s), and 6·18 (s) (OMe,NMe) and 7·58 (s, Ar*Me*), ν (KBr) 1715br.

TABLE 2

	0·039M	0·020M	0·010M
$CHCl_3$	2·79	2·79	2·79
OMe	6·38	6·38	6·38
endo-Me	7·86	7·86	7·85
	7·93	7·93	7·93
	7·96	7·96	7·95
exo-Me	8·18	8·17	8·15

Essentially complete conversion of the tricarboxylic acid into trimethyl 1,3-dimethylpyrrole-2,4,5-tricarboxylate occurred when the reaction time was extended to 15 h (0°).

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[28] A. H. Corwin and J. L. Straughn, *J. Amer. Chem. Soc.*, 1948, **70**, 1416.