Bile Pigments

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1 Introduction

Bile pigments are open-chain tetrapyrrolic compounds which are derived in Nature by oxidative degradation and ring-opening of the prosthetic groups of haemoproteins. In mammals, bile pigments are simply waste materials which are excreted, and they have no biological function. However, lower animals and plants possess bile pigments which serve important, and often vital, purposes. The protective colorations of some insects are bile pigments, as is the purple defensive secretion of the sea hare. Often, the bile pigments are protein-bound, especially in algae and in green plants; these chromoproteins possess photosynthetic activity, and one of the most important, phytochrome, is the photoreceptor for the photoregulation of growth in plants.

Several books[1] dealing with bile pigments and one substantial review[2] have been published. All of these are now somewhat dated and it is our intention to give a contemporary view of the position of research in this field, laying particular emphasis on the interaction between the chemical and biochemical disciplines. Literature received in England after 1st January 1975 has not been considered.

A. Nomenclature.—Trivial names abound in the natural series of bile pigments. Two systems for numeration are at present in use; the first, which will be used throughout this review because it is consistent with all of the early, and most of the current, literature, is shown in (1). This system also emphasizes the relationship between bile pigments and porphyrins, for which the Fischer system of nomenclature is still widely used. The second type of numeration is shown in (2); this system represents an attempt to accommodate all pyrrole-derived pigments within the corrin system of nomenclature. We have rejected this at the present time because few, if any, bile-pigment publications have used it.

The completely saturated tetrapyrrole (3) is termed 'bilinogen'. Pigments with one and two unsaturated interpyrrole links are termed 'bilienes' [*e.g.* (4)] and 'bilidienes' [*e.g.* (5), (6)] respectively. *a,c*-Bilidienes (6) are usually termed 'rubins'. The completely unsaturated pigment *a,b,c*-bilitriene (7) is called a

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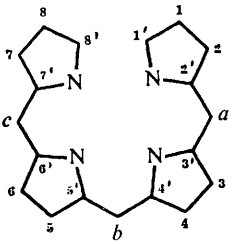
[1] (*a*) H. Fischer and H. Orth, 'Die Chemie des Pyrrols', Vol. IIi, Akademische Verlag., Leipzig, 1937; (*b*) R. Lemberg and J. W. Legge, 'Haematin Compounds and Bile Pigments', Interscience, New York, 1949; (*c*) C. H. Gray, 'The Bile Pigments', Methuen, London, 1953; (*d*) T. K. With, 'Bile Pigments', Academic Press, New York, 1968.

[2] R. Lemberg, *Rev. Pure Appl. Chem.* (*Australia*), 1956, **6**, 1.

'verdin' on account of the blue-green colour exhibited by this chromophore. The analogous compounds with terminal pyrrole rings in place of the pyrrolinone rings are called bilane, biladienes, and bilatriene; these compounds do not occur naturally but they do figure prominently as intermediates in porphyrin syntheses.[3]



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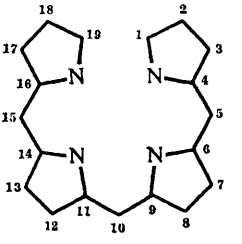

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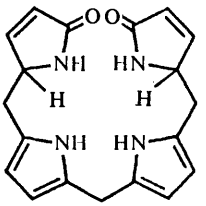

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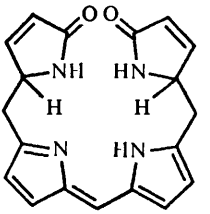

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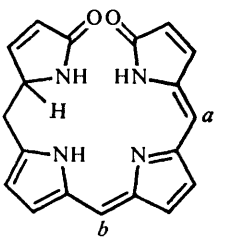
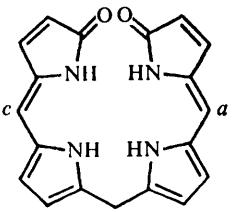


(5)

a,b-Bilidiene



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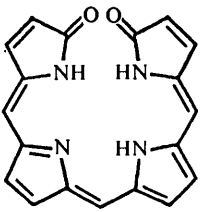


(7)

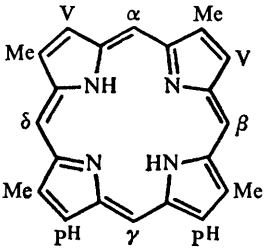
a,c- Bilidiene

Some bile pigments can be named on the basis of the porphyrin from which they are nominally derived. Thus, biliverdin-IXα (9) is the bile pigment obtained by rupture of protoporphyrin-IX (8) with oxidative removal of the α *meso*-carbon atom. Ring opening at the β, γ, or δ *meso*-carbon would give biliverdins-IXβ

[3] For a review see A. H. Jackson and K. M. Smith, in 'The Total Synthesis of Natural Products', Vol. 1, ed. J. W. ApSimon, Wiley, New York 1973.

(10), -IXγ (11), and -IXδ (12) respectively. As it happens, these pigments (9)—(12) have been obtained by oxidation of the iron complex of protoporphyrin-IX, but the scheme can also be used for any bile pigment, no matter what its origin, provided that the array of substituents can be related to that in a trivially named porphyrin.

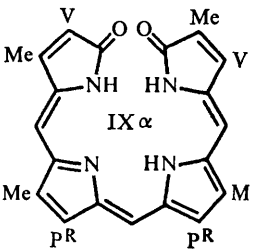
With only one exception (see later), all natural bile pigments have been shown to have the -IXα orientation of substituents. Therefore, except where otherwise stated, the -IXα isomer will be assumed in the ensuing discussion.



(8)

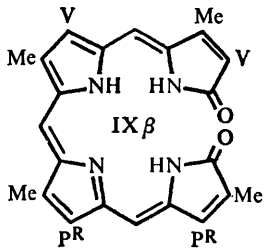
V = $-CH=CH_2$

$P^R = CH_2CH_2CO_2R$



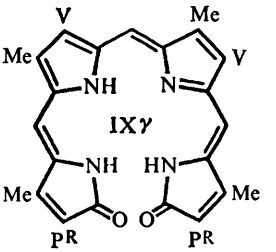
(9) R = H

(32) R = Me



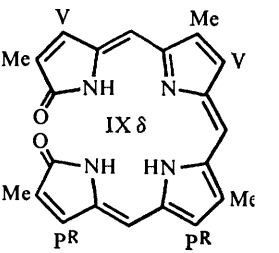
(10) R = H

(33) R = Me



(11) R = H

(34) R = Me

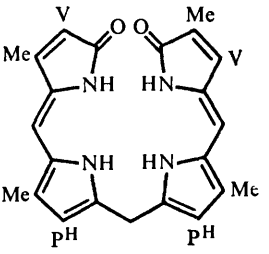


(12) R = H

(35) R = Me

B. Occurrence.—*In Animals.* Bile pigments have long been recognized as the waste products of haemoglobin catabolism in higher animals. The principal sites of haemoglobin breakdown are the reticuloendothelial cells of spleen, bone marrow, liver, and to a smaller extent, the kidneys. Rupture of the blood pigment, with loss of the α *meso*-carbon atom (as carbon monoxide[4]) and the iron atom (which is re-used), affords biliverdin-IXα (9). In man and mammals, biliverdin is reduced, giving bilirubin (13); the reduction is catalysed by biliverdin reductase, and the sites of highest activity of this enzyme are the spleen and liver, two of the principal sites of haem [iron(II) protoporphyrin-IX] breakdown.

[4] T. Sjoestrand, *Acta physiol. Scand.*, 1952, **26**, 328, 334, 338; *Ann. New York Acad. Sci.* 1970, **174**, 5; G. D. Ludwig, W. S. Blakemore, and D. L. Drabkin, *Biochem. J.*, 1957, **66**, 38P.



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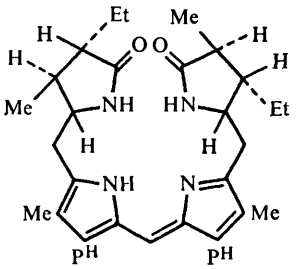

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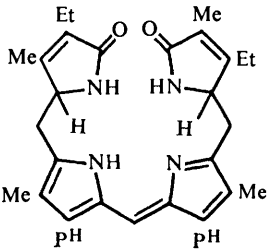


(15)

MO calculations[5] predict that biliverdin will have pronounced electron-acceptor properties because the lowest vacant molecular orbital is a bonding molecular orbital. The central (*b*) methine bridge is predicted to be particularly electron-deficient, and this accounts for reduction at this site; furthermore, the loss in resonance energy is only *ca.* 1 kcal mol^{-1} compared with *ca.* 8 kcal mol^{-1} if reduction takes place at the *a* or *c* positions.

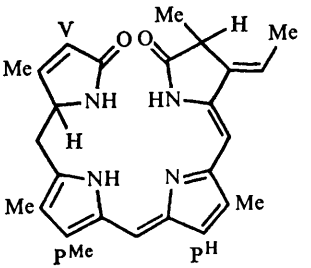
Bilirubin is converted ('conjugated') into its water-soluble diglucuronide salt which is excreted with the bile into the duodenum, but hydrolysis in the intestinal tract regenerates free bilirubin, which is reduced by intestinal bacteria to give urobilinoid chromogens as the final products.[6] These colourless compounds (bilinogens) produce orange-yellow urobilinoids such as stercobilin (14) and urobilin (15). Both stercobilin and *d*-urobilin are optically active, but *i*-urobilin is inactive. *d*-Urobilin is often found[7] after administration of broad-spectrum antibiotics such as tetracycline. Under normal conditions a complex mixture of bile pigments is excreted in the faeces; the characteristic yellow skin pigmentation found in jaundice is caused by retention of bilirubin. The most common source of bilirubin is, however, ox gallstones, in which the bile pigment is found as the pure calcium salt.

[5] B. Pullman and A.-M. Perault, *Proc. Nat. Acad. Sci. U.S.A.*, 1959, **45**, 1476.
[6] C. J. Watson, *Ann. Internal Medicine*, 1969, **70**, 839.
[7] C. J. Watson and P. T. Lowry, *J. Biol. Chem.*, 1956, **218**, 633.

Biliverdin. This green pigment is found in dog placenta,[8] in the integumental cells and haemolymphs of insects such as[9] the praying mantis, locust, and grasshopper, in the egg shells of some birds,[10] in the bones, fins, and skin of certain fish,[11] in the blue coral *Heliopora coerulea*,[12] and in the root nodules of some plants.[13] In addition, biliverdin-IXγ (11), the only natural bile pigment so far to be shown[14] not to have the -IXα substituent orientation, occurs in the integumental cells of the caterpillar of the Cabbage White butterfly.

Aplysioviolin. This is the principal pigment in the purple defensive secretion of the sea hare *Aplysia.* The pigment has been characterized[15] as (16) and because the sea hare contains no significant amounts of porphyrin pigment it appears likely that the bile pigment comes from the biliprotein of red algae which the hare ingests with its food.

Phycoerythrobilin. This pigment is obtained from the chromoproteins of red and blue-green algae. It is cleaved from the protein under relatively mild conditions[16] and has been assigned[17] structure (17).



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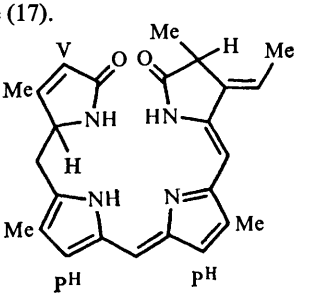
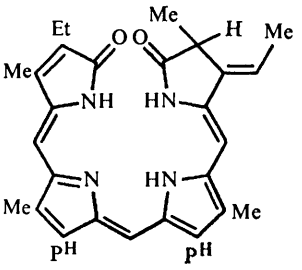


(17)

Phycocyanobilin. Phycocyanin is the photosynthetic biliprotein found in blue-green algae and the prosthetic pigment is called phycocyanobilin. Its structure has been defined[18] as (18).

[8] R. Lemberg and J. Barcroft, *Biochem. J.*, 1934, **28**, 978.
[9] (*a*) M. Passama-Vuillaume, *Bull. Soc. Zool. France*, 1965, **90**, 485; (*b*) W. Rudiger, *Angew Chem. Internat. Edn.*, 1970, **9**, 473.
[10] R. Lemberg, *Annalen*, 1931, **488**, 74.
[11] D. L. Fox and N. Millott, *Experientia*, 1954, **10**, 185; W. Rüdiger and L. Abolins, *ibid.*, 1966, **22**, 298; 1969, **25**, 574.
[12] W. Rudiger, W. Klose, B. Tursch, N. Houvenaghel-Crevecour, and H. Budzikiewicz. *Annalen*, 1968, **713**, 209; W. Rüdiger, W. Klose, M. Vuillaume, and M. Barbier, *Experientia*, 1968, **24**, 1000.
[13] A. I. Virtanen and J. K. Mietten, *Acta Chem. Scand.*, 1949, **3**, 17.
[14] W. Rudiger, in 'Porphyrins and Related Compounds', Biochemical Society Symposium No. 28, ed. T. W. Goodwin, Academic Press, London, 1968, p. 121.
[15] W. Rüdiger, *Z. physiol. Chem.*, 1967, **348**, 1554; see also ref. 14.
[16] C. O'hEocha, P. O'Carra, and D. M. Carroll, *Biochemistry*, 1964, **3**, 1343; Y. Fujita and A. Hattori, *J. Biochem.* (*Japan*), 1962, **51**, 89; *J. Gen. Appl. Microbiol.*, 1963, **9**, 253; C. O'hEocha and P. O'Carra, *Phytochemistry*, 1966, **5**, 993.
[17] (*a*) D. J. Chapman, H. W. Siegelman, and W. J. Cole, in ref. 14, p. 107; (*b*) W. Rudiger, P. O'Carra, and C. O'hEocha, *Nature*, 1967, **215**, 1477; (*c*) H. L. Crespi, L. J. Boucher, G. Norman, J. J. Katz, and R. C. Dougherty, *J. Amer. Chem. Soc.*, 1967, **89**, 3642.
[18] D. J. Chapman, H. W. Siegelman, and W. J. Cole, *J. Amer. Chem. Soc.*, 1967, **89**, 3643; see also ref. 17 (*b*).

Phytochrome. Phytochrome exists in two forms, 'P_r' (724 nm) and 'P_{fr}' (665 nm), which are photochemically interconvertible. It exists in all higher plants, and in red and green algae, and acts as a photoreceptor for the regulation of growth and development in plants. Structural investigations have been hampered by the difficulties involved in obtaining large enough quantities of the pigment; a structure (19), which is not yet generally accepted, has been proposed for it.[17a]



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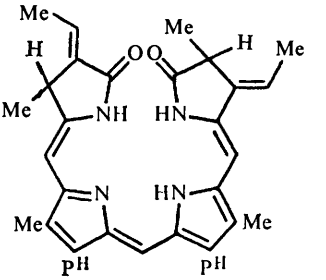


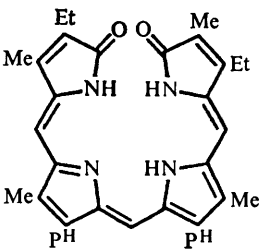
(19)

2 Structure Elucidation

A. Intuitive Methods.—Much of the early structural work carried out in Germany relied heavily on intuition and application of experience already gained in the porphyrin field. The colours of bile pigments were particularly helpful in obtaining a reasonable idea of gross structure; bilinogens (3) are, of course, colourless, bilienes (4) are yellow, *a,b*-bilidienes (5) are violet, *a,c*-bilidienes (6) are yellow-red, and bilitrienes (7) are blue or green. Fischer considered the bile pigments to be tetrapyrrylethylenes for many years, but in 1931 he applied[19] a logically pointless but intuitively superb reaction (Section 2B) to bilirubin, and from this he reasoned the structure of bilirubin.[1a] At first, a symmetrical arrangement of the side-chains was assumed, but further work soon revealed[20] that the substituent pattern is the same as in protoporphyrin-IX (8); the ramifications of this latter discovery must have been tremendously exciting and led to the initiation of a massive synthetic effort by the Munich school under the direction of Fischer and Siedel, in which a host of bile pigments of both natural and purely synthetic interest were prepared and identified.

Once a certain amount of ground work had been done it became possible to identify new bile pigments on the basis of interconversion of these with known, fully characterized, bile pigments. This was not always completely successful; *e.g.*, Lemberg and Bader[21] obtained a pigment (phycoerythrobilin?) from phycoerythrin by alkaline hydrolysis, and identified it to be mesobiliverdin (20). At that time it was not realized that alkali induces irreversible prototropic shifts in phycoerythrobilin (17), the end product of which is mesobiliverdin (20). On

[19] H. Fischer and R. Hess, *Z. physiol. Chem.*, 1931, **194**, 193.
[20] W. Siedel and H. Fischer, *Z. physiol. Chem.*, 1933, **214**, 145.
[21] R. Lemberg and G. Bader, *Annalen*, 1933, **505**, 151.



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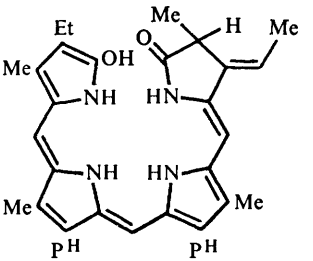
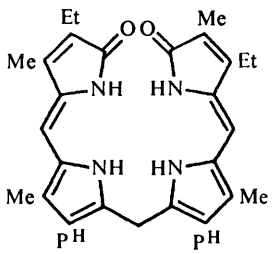


(21)

the other hand, Rudiger *et al.*[17b] were able to challenge the structure (21) proposed for phycocyanobilin by Crespi *et al.*[17c] on the basis that it was not compatible with the ready isomerization of phycocyanobilin (18) into mesobiliverdin (20).

B. Degradative Methods.—*Non-oxidative Degradation.* Before the full structure for bilirubin had been elucidated it was realized that the molecule contained vinyl substituents. Thus, Fischer applied[19] to bilirubin the resorcinol fusion method[22] by which vinyl groups are normally removed from porphyrins, in the hope of preparing a novel de-vinyl (deutero)bilirubin. Only inconclusive results were obtained but, unaccountably, Fischer was moved to apply the resorcinol fusion to mesobilirubin (22), in which the vinyl groups are replaced by ethyl functions, which could not be expected to be removed in the melt. This gave a mixture of isoneoxanthobilirubic acid (23) and neoxanthobilirubic acid (24);[20] this told Fischer that breakdown of bilirubin had occurred either side of a methane carbon linking two oxopyrromethene moieties.† Once the oxopyrromethene had been identified as a mixture of the two isomers (23) and (24), the structure for bilirubin followed logically.



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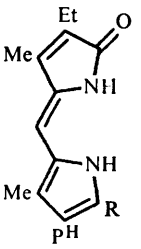
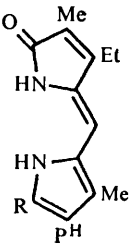


(23) R = H
(28) R = Me



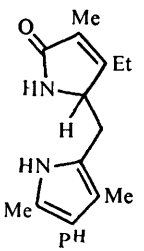
(24) R = H (53) R = CHO
(27) R = Me (54) R = CH_2OH

†Terminal rings of bile pigments exist as the lactam tautomer;[23] almost all of the early literature used the lactim form for these rings and the corresponding rings in the dipyrrolic degradation products.

[22] O. Schumm, *Z. physiol. Chem.*, 1928, **178**, 1.

[23] C. H. Gray, D. C. Nicholson, and R. A. Nicolaus, *Nature*, 1958, **181**, 183.

Mild reduction of bilirubin with hydriodic acid in acetic acid yields[24] chiefly bilirubic acid (25) with some of its isomer (26), and oxidation of these with alkaline permanganate affords the orange-yellow oxopyrromethenes xantho-bilirubic acid (27) and isoxanthobilirubic acid (28).[25]



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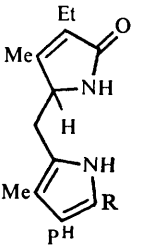
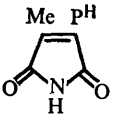


(26) R = Me

(60) R = CHO



(29)

Oxidative Degradation. (For details of photo-oxidation, see Section 5D). With chromic acid, bilirubin yields haematinic acid (29), a compound similarly obtained from haematin, and which allowed the very early workers to deduce that the porphyrins and bile pigments were in some way related.

Nicolaus[26] has described a more controlled oxidative procedure, using alkaline permanganate, which can be applied to bile pigments on a micro-scale. Pyrrole-2,5-dicarboxylic acids are produced from the two internal rings of the bile pigment, and these can be identified by paper chromatography in quantities below 5 μg. The most well-known application of this procedure to bile-pigment chemistry was the demonstration[27] that the biliverdin obtained from chemical ('coupled') oxidation of haemin [iron(III) protoporphyrin-IX chloride] is a mixture of all four possible biliverdin isomers [(9)—(12)] and not just biliverdin-IXα as had been claimed by Lemberg.[2] Clearly, using the Nicolaus procedure, biliverdin-IXα can only yield one pyrrole-2,5-dicarboxylic acid [*i.e.* (30)]. However, Gray *et al.*[27] showed that significant quantities of the pyrrole (31) were obtained from the degradation, and reached the inescapable conclusion that some of the bile pigment from the coupled oxidation of haemin must have methyl–vinyl substituents sited on the two internal rings. All four biliverdins have since been separated[28,29] as the dimethyl esters (32)—(35) (see p. 387).

[24] H. Fischer and H. Rose, *Z. physiol. Chem.*, 1912, **82**, 391; *Ber.*, 1912, **45**, 1579; O. Piloty and S. J. Thannhauser, *Annalen*, 1912, **390**, 191; *Ber.*, 1912, **45**, 2393; O. Piloty, *ibid.*, 1913, **46**, 1000.

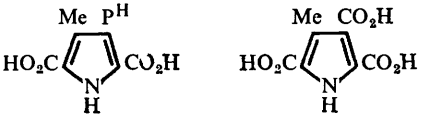
[25] Ref. 1 (*a*), p. 121.

[26] R. A. Nicolaus, *Rass. Med. Sper.*, 1960, **7**, Suppl. 2.

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

[28] R. Bonnett and A. F. McDonagh, *J.C.S. Perkin I*, 1973, 881.

[29] P. O'Carra and E. Colleran, *J. Chromatog.*, 1970, **50**, 458.



(30) (31)

The permanganate method has the disadvantage that it yields only information on the nature of the two internal rings, and this was clearly not enough for structural elucidation of the algal bile pigments. Most pyrrolic compounds are transformed into maleimides on treatment with chromic acid; its use as a degradative reagent has been adapted by Rüdiger.[14] Table 1 shows the oxidizing potential of the reagent to be pH-dependent. The comparative rates of oxidation of bile pigments and protoporphyrin-IX are shown in Table 2; biliverdin is oxidized approximately 100 times faster than protoporphyrin-IX, and bile pigments with unsaturated side-chains degrade much faster than the corresponding ones with saturated substituents.

Table 1 *Oxidation of biliverdin at different pH's*[14]

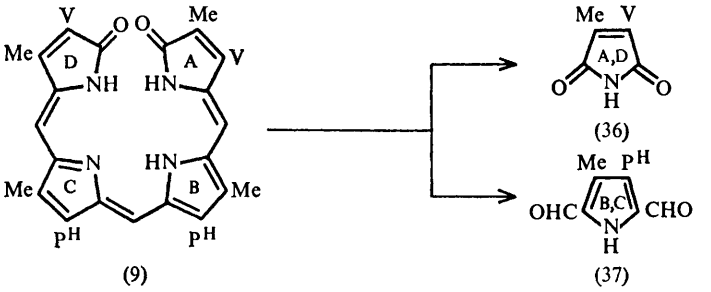
pH	$t_{\frac{1}{2}}$
1.7	4.5 min
3.1	52 min
4.1	7 h
5.1	24 h
5.9	29 h

Table 2 *Oxidation of tetrapyrroles with chromic acid at pH 1.7*[14]

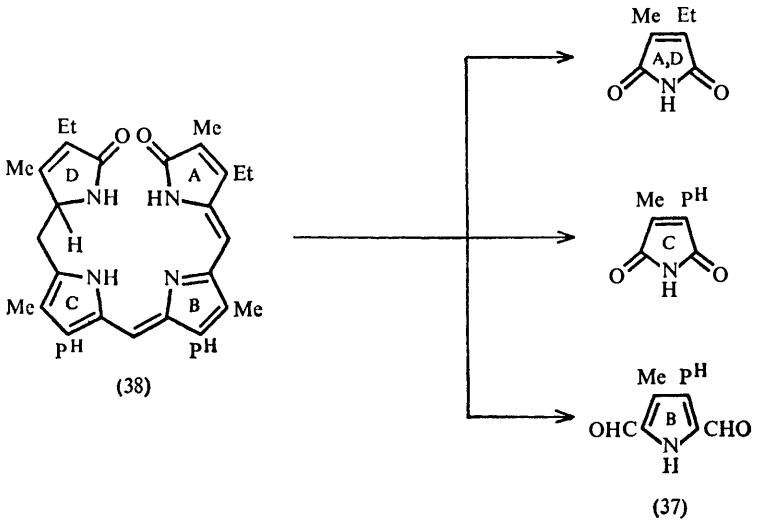
Pigment	$t_{\frac{1}{2}}$
Aplysioviolin (16)	0.5 min
Mesobiliverdin (20)	16 min
Urobilin (15)	20 min
Protoporphyrin-IX (8)	400 min

The chromic acid degradation is usually carried out under two sets of conditions: (i) chromium trioxide in 1M-H_2SO_4—'chromic acid degradation', or (ii) sodium dichromate in 1% sodium bisulphate (pH 1.7)—'chromate degradation'. If the degradation is carried out at 20 °C any ester linkage present remains intact in the products; at 100 °C the esters are hydrolysed. The significant advantage of this procedure is that vinyl substituents survive to yield maleimides bearing vinyl groups.

Chromate degradation of biliverdin (Scheme 1) yields 3-methyl-4-vinylmaleimide (36) in good yield from the terminal rings (A and D), but the middle rings (B and C) do not give maleimides in good yield. Instead, these rings give a 2,5-diformylpyrrole (37), which is oxidized in more acidic media (such as that found in 'chromic acid degradation') to the corresponding maleimide. The



Scheme 1

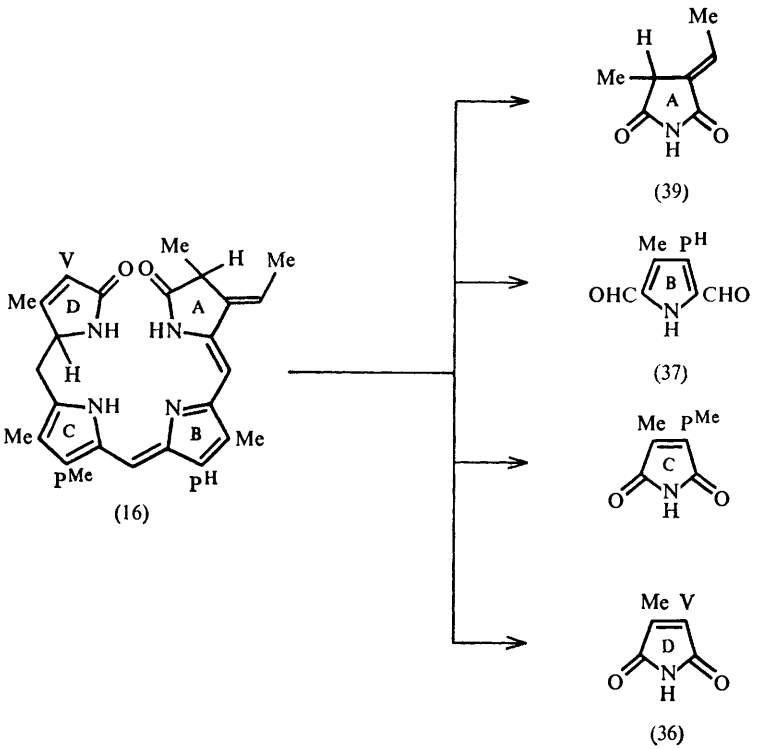


Scheme 2

2,5-diformylpyrrole can only be obtained from the middle rings (B and C) since they bear the appropriate methine carbons. Rüdiger[14] has shown also that with bilitrienes (7) the diformylpyrroles are produced from both of the inner rings, but with *a,b*-bilidienes (5) [*e.g.* mesobiliviolin (38) (Scheme 2)] the 2,5-diformylpyrrole (37) is derived almost exclusively from ring B.

Thus, by degradation and application of chemical intuition, it is possible to establish the sequence of substituents in a given bile pigment. The degradation products can be separated by thin-layer chromatography on silica gel, and the maleimides can be detected by the chlorine–benzidine reagent,[30] which is sensitive but non-specific. Complete identification can be achieved by comparison with authentic maleimides or by mass spectrometry. The 2,5-diformylpyrroles can be detected by the 2,4-dinitrophenylhydrazine reagent.

[30] (*a*) W. Rüdiger, *Z. physiol. Chem.*, 1969, **350**, 1291; (*b*) F. Reindel and W. Hoppe, *Chem. Ber.*, 1954, **87**, 1103.



Scheme 3

The technique can be carried out actually on a silica gel thin-layer plate. After spotting the bile pigment onto the base line, the chromic acid reagent is spotted on top of it; subsequent development of the plate in the normal way, followed by detection with the chlorine–benzidine reagent, allows identification of the constituent rings of the bile pigment.

Chromate oxidation[14] of aplysioviolin [(16); Scheme 3] yielded two imides, one 2,5-diformylpyrrole, and methyl ethylidene succinimide (39). Since the propionic acid side-chain was not esterified in the dialdehyde, the methyl ester (identified earlier) must have been on the middle ring, having a saturated link to one of the terminal pyrrolinone rings (see above); assumption of the -IXα substituent orientation led[14] to the proposal of structure (16) for aplysioviolin. Similarly, the products from oxidation of the pigment from the integumental cells of the caterpillar of the Cabbage White butterfly, showed[14] that this pigment could not be a -IXα type, and led to its identification as biliverdin-IXγ.

Another great advantage of the chromic acid degradation is that it can be carried out on the purified biliprotein itself.[14] R-Phycoerythrin, C-phycoerythrin (the letters refer to the source and properties of the biliprotein, see ref. 14),

and the pigments derived from these yield haematinic acid, methyl ethylidene succinimide (39) and methyl vinylmaleimide (36) upon chromic acid oxidation; since the phycoerythrobilins contain two carboxylic acid groups, the haematinic acid must be derived from two rings of the pigment.

Chromic acid degradation of C-phycocyanin and the pigment from this chromoprotein gave[17b] methyl ethylidene succinimide, methyl ethylmaleimide, and haematinic acid. Since C-phycocyanin on hydrolysis with alkali yields mesobiliverdin (20) (and caused Lemberg and Bader[21] to assume that phycocyanobilin and mesobiliverdin were one and the same compound) it follows that phycocyanobilin has the -IXα orientation pattern; this led to the proposal[17b] of structure (18) for phycocyanobilin. It is worth mentioning that if a degradative procedure which can be applied to both the chromoprotein and the isolated pigment had been available in Lemberg's day then progress would have been much more rapid in the field of algal bile pigments and he would not have been satisfied with the mesobiliverdin proposal for phycocyanobilin.

Having established the nature of the chromophores of the algal bile pigments, the chromic acid degradation method was also able to offer some information on the bonding of these to their apoproteins. Depending upon the conditions, chromic acid degradation of the native chromoproteins gave different results. For instance, C-phycocyanin at 100 °C is hydrolysed to give peptides which pass into solution, allowing isolation of methyl ethylmaleimide, haematinic acid, and methyl ethylidene succinimide; these same imides are obtained from free phycocyanobilin. When the oxidation is carried out at 20 °C the protein is not hydrolysed and remains intact. Whereas the same quantity of methyl ethylmaleimide can be extracted, only half of the expected amount of haematinic acid and no methyl ethylidene succinimide are obtained. The missing imide (39) and the other half of the haematinic acid can be liberated by hydrolysis without further quantities of oxidant being added.[14] The same result is also obtained with R-phycocyanin and, in principle, with R- and C-phycoerythrin. These experiments serve to illustrate that two of the rings of the bile pigment are linked to the protein. It is not known which functional groups in the protein are involved in these linkages, and this information can only come after the amino-acid sequences of these biliproteins have been determined.

Which of the two inner pyrrole rings is bound to protein has been established for phycoerythrin.[9b,14] On chromate oxidation at 20 °C the 2,5-diformylpyrrole (37) is liberated, while haematinic acid (29) is liberated only upon hydrolysis of the protein residue. Since the 2,5-diformylpyrrole can only be formed from ring B of *a,b*-bilidienes, this ring must be free and ring C must be bound to the protein in phycoerythrin.

3 Synthesis of Bile Pigments

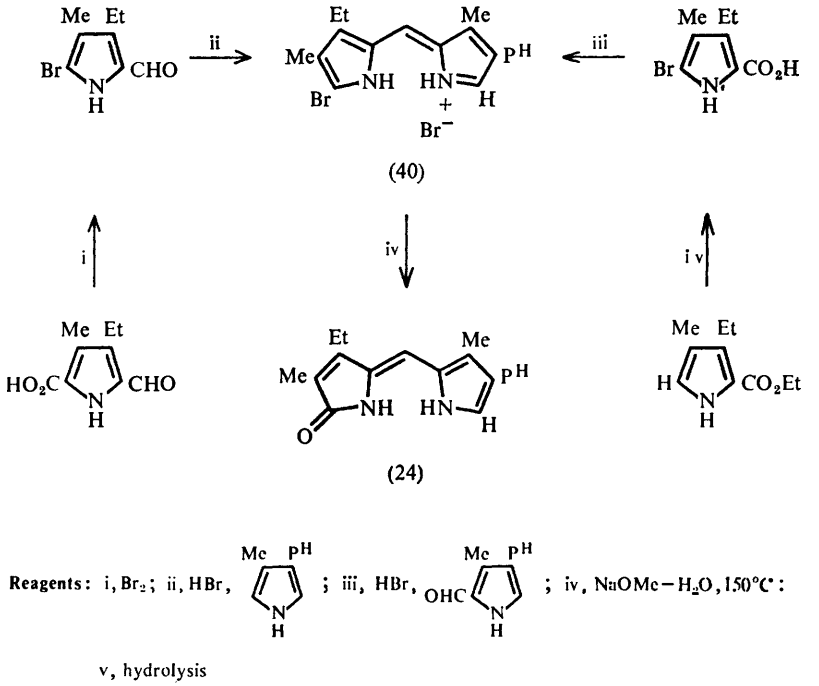
Bile pigments can be prepared either from pyrrolic compounds directly or else by ring-opening of porphyrins. In this section we shall deal only with the former approach; the latter method is discussed in Section 4.

Synthetic methods have been discussed in full elsewhere[3] and so only a brief

discussion will be given. However, the importance of total synthesis in the bile-pigment area should not be under-estimated because it was on the basis of this, and comparison with authentic compounds, that the early workers in Munich were able to confirm structural proposals and thereafter to piece together the complex relationships between the various pyrrole-derived pigments.[1a]

All direct approaches to bile pigments proceed by condensation of two oxopyrromethenes, oxopyrromethanes, or of an oxopyrromethene and an oxopyrromethane. In a few cases, 5-bromopyrromethenes have been self-condensed, but the terminal bromo-functions are hydrolysed either during or shortly after the condensation, and the bile pigments are isolated with the terminal lactam rings present.

Synthesis of Oxopyrromethenes and Oxopyrromethanes.—A typical oxopyrromethene is neoxanthobilirubic acid (24), and two slightly different approaches[31a,b] are presented in Scheme 4. These employ classical pyrrole chemistry

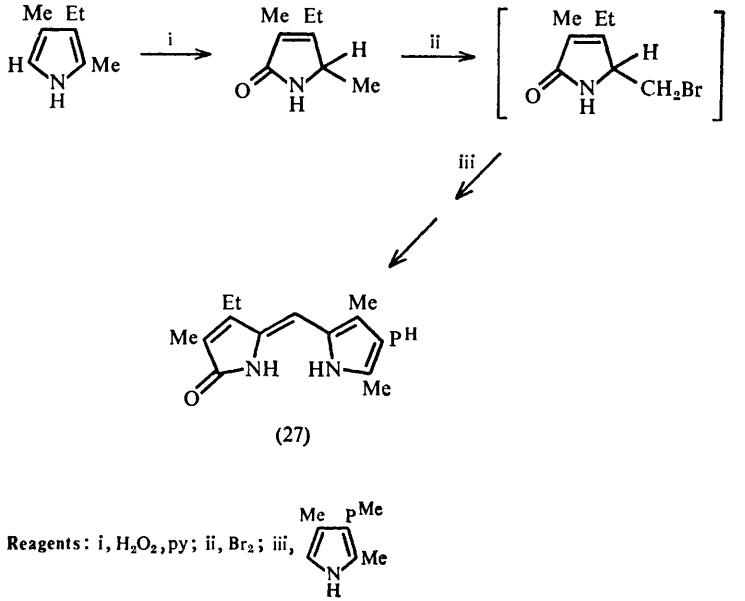


Scheme 4

which should need no discussion. It is not essential to introduce the lactam function by hydrolysis of a 5-bromopyrromethene [*e.g.* (40)]. If required, the oxygen function can be carried through from a pyrrolinone; such an approach

[31] (*a*) W. Siedel and H. Fischer, *Z. physiol. Chem.*, 1933, **214**, 146, 163; (*b*) W. Siedel, *Z. physiol. Chem.*, 1935, **231**, 181, 197.

is outlined in Scheme 5, which shows the synthesis of xanthobilirubic acid (27). The pyrrolinone is obtained by hydrogen peroxide oxidation of a 2-unsubstituted pyrrole. Bromination and condensation with the resulting (not isolated) bromomethylpyrrolinone of a 2-unsubstituted pyrrole affords the required product.



Scheme 5

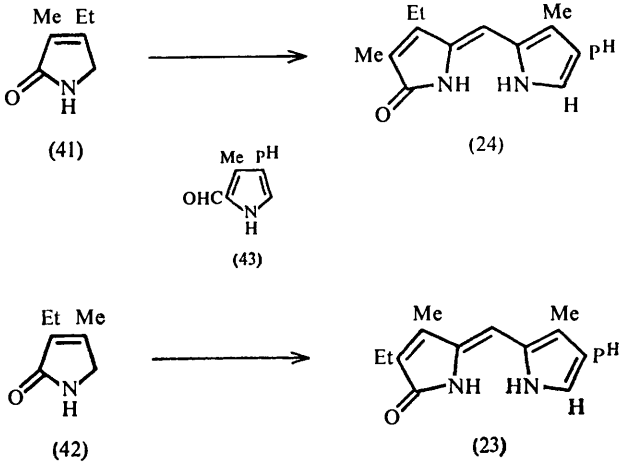
Yet another variation is possible. Scheme 6 shows the synthesis[32] of neoxanthobilirubic acid (24) and isoneoxanthobilirubic acid (23) by condensation of 4-ethyl-3-methyl- (41) or 3-ethyl-4-methyl-pyrrolin-2-one (42) with 2-formyl-3-methylpyrrole-4-propionic acid (43).

Gossauer[33] has recently reported an ingenious approach to an oxopyrromethene (44) required for his synthesis of phycocyanobilin. The two rings (Scheme 7) were joined by way of a sulphur-extrusion reaction.

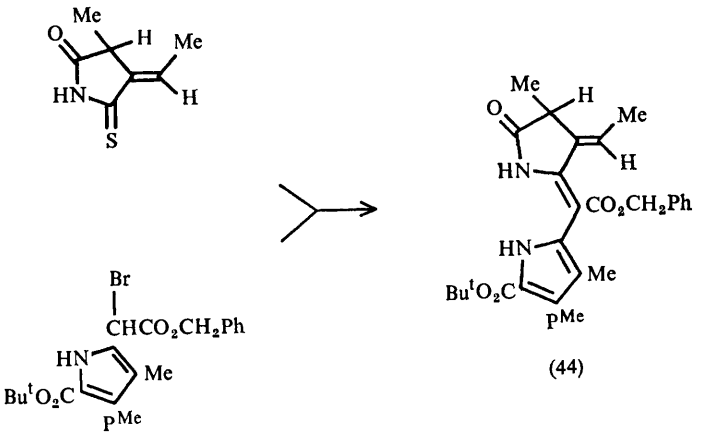
Only one direct approach to oxopyrromethanes has been described; Johnson and co-workers found that peroxide oxidation of 5-unsubstituted pyrromethanes affords[34] oxopyrromethanes (45) in moderate yield. The most widely used method for synthesis of oxopyrromethanes is reduction of the corresponding oxopyrromethene, either with sodium amalgam or by hydrogenation over a de-activated palladium catalyst.

Many bile pigments possess chiral centres and are thus optically active. A typical example is stercobilin (14). The building blocks for such bile pigments

[32] H. Plieninger and U. Lerch, *Annalen*, 1966, **698**, 196.
[33] A. Gossauer and W. Hirsch, *Tetrahedron Letters*, 1973, 1451; *Annalen*, 1974, 1496.
[34] J. H. Atkinson, R. S. Atkinson, and A. W. Johnson, *J. Chem. Soc.*, 1964, 5999.



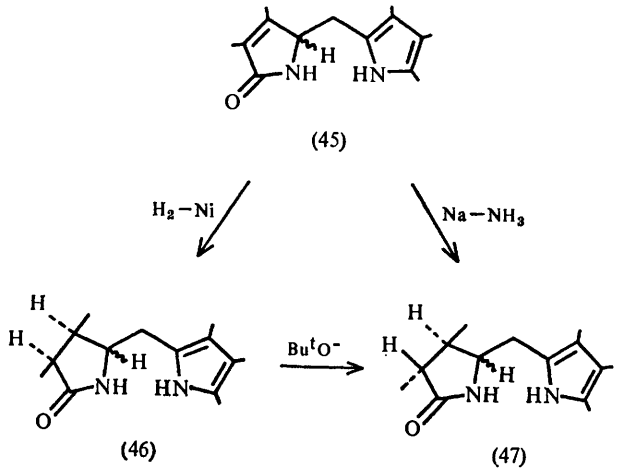
Scheme 6



Scheme 7

must, of necessity, contain the chiral carbon atoms assembled in the correct stereochemical array. Plieninger and his co-workers have made critically important contributions in this area. Hydrogenation of an oxopyrromethane (45) at high temperature and pressure in the presence of Raney nickel furnishes the *cis*-reduced compound (46) (Scheme 8).[32] On the other hand, if the reduction is carried out with sodium in liquid ammonia, the *trans*-isomer (47) is obtained. The *trans*-compound can also be obtained by epimerization of the *cis*-isomer with strong base, such as t-butoxide in t-butyl alcohol. Using these intermediates, Plieninger and co-workers[35] have carried out a whole series of sophisticated syntheses of stercobilin and other optically active urobilinoids (see also ref. 3).

[35] H. Plieninger and J. Ruppert, *Annalen*, 1970, **736**, 43; H. Plieninger, K. Ehl, and A. Tapia, *ibid.*, p. 62.

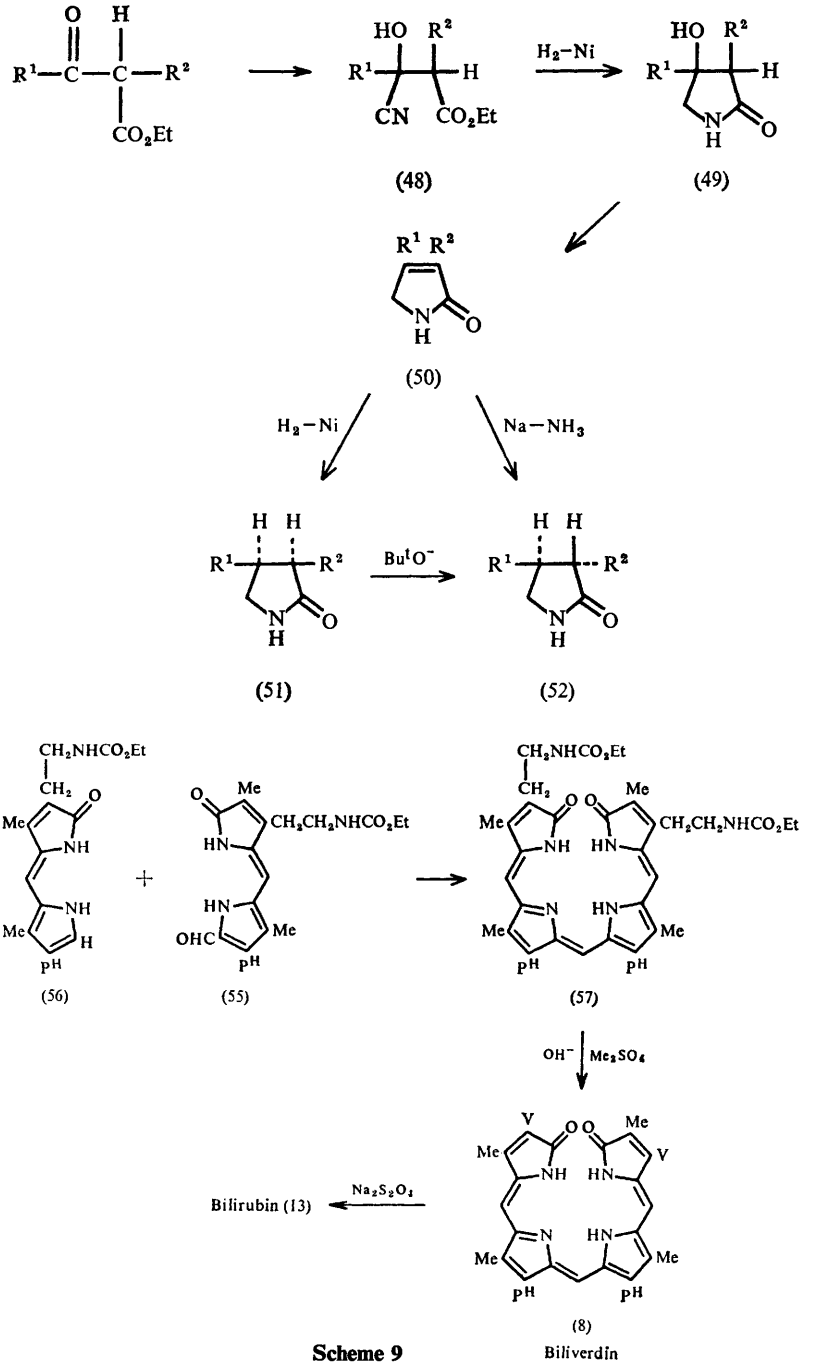


Scheme 8

Pyrrolinones.—As mentioned earlier, pyrrolinones can be obtained[34] by oxidation of 2-unsubstituted pyrromethanes with hydrogen peroxide in pyridine (*cf.* ref. 36). Contemporary synthetic approaches to bile pigments have tended to rely more upon ring synthesis of pyrrolinones. Perhaps the most widely used method is that of Plieninger.[37] Catalytic hydrogenation (Raney nickel) of cyanohydrins (48) obtained from β-keto-esters gives a diastereoisomeric mixture of hydroxypyrrolidones (49), which can be dehydrated to give the required pyrrolinones (50). It is interesting that reduction of (50) with Raney nickel or sodium in liquid ammonia gives the *cis*- and *trans*-pyrrolidones (51) and (52) respectively (*cf.* oxopyrromethanes) and that (51) can be converted into (52) by treatment with t-butoxide.[32] Pyrrolinones can also be obtained *via* an internal Emmons reaction.[38]

Condensations to give Bile Pigments.—Treatment of formyl neoxanthobilirubic acid (53) [obtained by the Gatterman reaction from neoxanthobilirubic acid (24)] with isoneoxanthobilirubic acid (23) gives[39] mesobiliverdin (20). Reduction of the aldehyde group in (53) to give the corresponding hydroxymethyl derivative (54) followed by coupling with isoneoxanthobilirubic acid (23) similarly gives[40] mesobilirubin; these facts firmly established the constitutions of biliverdin and bilirubin.[1a]

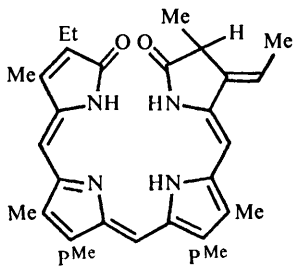
[36] H. Fischer, T. Joshioka, and P. Hartmann, *Z. physiol. Chem.*, 1932, **212**, 146.
[37] H. Plieninger and M. Decker, *Annalen*, 1956, **598**, 198; H. Plieninger, and J. Kurze, *ibid.*, 1964, **690**, 60.
[38] G. Stork and R. Matthews, *Chem. Comm.*, 1970, 445.
[39] W. Siedel, *Z. physiol. Chem.*, 1935, **237**, 8.
[40] W. Siedel, *Z. physiol. Chem.*, 1937, **245**, 257.



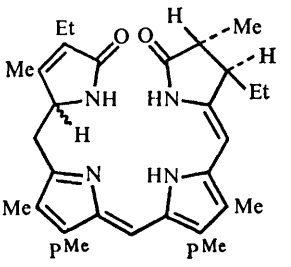
Scheme 9

Total syntheses of bilirubin (13) and biliverdin (8) were complicated by the presence of the vinyl groups in these molecules. Although vinyl-substituted pyrrolinones are now accessible,[41,42] Fischer's pioneering work made use of urethane protecting groups (obtained by Schmidt degradation of propionic side-chains). Thus (Scheme 9)[43] condensation of the formyl-oxopyrromethene (55) with the 5-unsubstituted oxopyrromethene (56) afforded the bilitriene (57). Hydrolysis and exhaustive methylation generated the vinyl groups in biliverdin (8). Reduction with hydrosulphite then gave bilirubin.

Slight modifications of this concept have been used in the synthesis[33] of phycocyanobilin dimethyl ester (58) using an ethylidene-substituted oxopyrromethene, in the synthesis[44] of mesobilirhodin dimethyl ester (59) from an

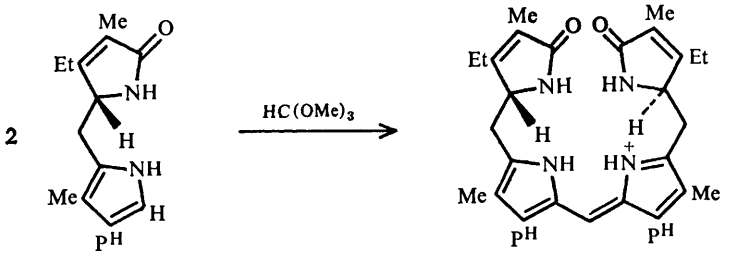


(58)



(59)

oxopyrromethene and an oxopyrromethane, and in the synthesis of optically active[36] urobilin (15). In the last case, a derivative of neobilirubic acid (25) was resolved, and the (+)- and (−)-enantiomers were condensed separately with racemic formyl isoneobilirubic acid (60). The two diastereomeric mixtures were crystallized three times to give two urobilin-IXα hydrochlorides {$[\alpha]_D^{20}$ +4500° and −4800°}, presumably the *RR*- and *SS*-enantiomers of (15).

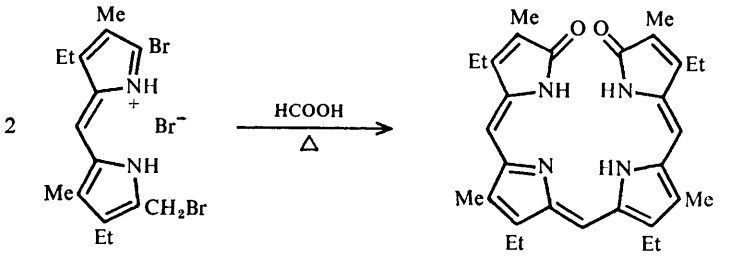


(+) - Urobilin - XIII α salt

Scheme 10

[41] H. Plieninger and R. Steinstrasser, *Annalen*, 1969, **723**, 149.
[42] H. Plieninger, K.-H. Hentschel, and R.-D. Kohler, *Annalen*, 1974, 1522.
[43] H. Fischer and H. Plieninger, *Z. physiol. Chem.*, 1942, **274**, 231.
[44] A. Gossauer and D. Miehe, *Annalen*, 1974, 352.

Treatment of oxopyrromethenes or oxopyrromethanes (Scheme 10) with a suitable one-carbon unit produces symmetrically substituted bile pigments. A useful route to relatively large quantities of a model bilitriene, aetiobiliverdin-IVγ, has been described.[45] Heating of the readily available 5-bromo-5′-bromomethylpyrromethene hydrobromide (61) in wet formic acid causes 'tail to tail' self-condensation and hydrolysis of the bromo-function to give aetiobiliverdin-IVγ (62). The product is contaminated with aetioporphyrin-I, produced by 'head to tail' self-condensation, and this can be removed by column chromatography.



(61)

Aetiobiliverdin – IVγ

(62)

4 Formation of Bile Pigments

A. Mammalian Bile Pigments.—The formation of bilirubin from haemoglobin has been known for well over a century; *e.g.* in 1847 Virchow[46] described the formation of 'haematoidin' crystals in blood extravasates, but it was not until 1923 that Fischer and Reindel[47] proved haematoidin to be identical with bilirubin (13). After a considerable amount of further work, the final elegant proof that bilirubin is formed by breakdown of the blood pigments was provided, in 1950, by London *et al.*,[48] who administered [^{15}N]glycine intravenously and orally to adults, and found that up to 90% of faecal bile pigments could be accounted for by destruction of erythrocytes. Most of the labelled erythrocytes were destroyed after about 100—140 days, indicating that mature circulating erythrocytes have an average life span of about 120 days.

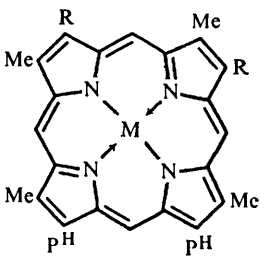
The classical theory of bile-pigment formation[49] summarized in Scheme 11 proposes separation of haem (63) from the protein matrix, followed by loss of iron from 'haematin' (64) to give free protoporphyrin-IX (8), which then undergoes oxidative rupture and reduction to give bilirubin (13). Despite poor experimental support, this view was accepted for very many years, even though the

[45] K. M. Smith, *J.C.S. Perkin I*, 1972, 1471.
[46] R. Virchow, *Arch. Path. Anat. u. Physiol.* (*Virchow's*), 1847, **1**, 379.
[47] H. Fischer and F. Reindel, *Z. physiol. Chem.*, 1923, **127**, 299.
[48] I. M. London, R. West, D. Shemin, and D. Rittenberg, *J. Biol. Chem.*, 1950, **184**, 351.
[49] M. Nencki and N. Sieber, *Arch. Exp. Pharmakol.*, 1884, **18**, 401; *ibid.*, 1888, **24**, 430; M. Nencki and J. Zaleski, *Z. physiol. Chem.*, 1900, **30**, 384; *Ber.*, 1901, **34**, 997.

$$\text{Haemoglobin} \xrightarrow{-\text{globin}} \underset{(64)}{\text{Haematin}} \xrightarrow{-\text{iron}} \underset{(8)}{\text{Protoporphyrin-IX}} \xrightarrow[\text{redn.}]{\text{Oxidn.-}} \underset{(13)}{\text{Bilirubin}}$$

Scheme 11

direct conversion of protoporphyrin-IX into bilirubin had never been demonstrated. In 1951, London *et al.* did report[50] that ^{14}C-labelled protoporphyrin-IX gave rise to activity in faecal bile pigments several days after injection but these authors suggested that iron may have been introduced into the porphyrin before breakdown took place, and they did not claim this experiment to be proof of the classical theory. The intermediacy of haematin (64) in bile-pigment formation has been a subject of much debate; in cases of haematin jaundice (a condition where haematin accumulates in the plasma without an increase in the serum bilirubin level), Schöttmuller[51] and Schumm[52] concluded that haematin was not a normal precursor of bilirubin, and this view was reinforced[53] by the observation that intravenously injected haematin does not cause an increase in the excretion of bilirubin. Haematin was therefore thought of as a side-product in haemoglobin breakdown. Lemberg[54] injected mesohaematin (65) into rabbits and found that



(63) M = Fe^{II} ; R = V

(64)† M = Fe^{III}—OH ; R = V

(65)† M = Fe^{III}—OH ; R = Et

(66) M = py → Fe^{II} ← py ; R = V

(75) M = Fe^{III}—Cl ; R = V

(76) M = Fe^{III}—Cl ; R = H

(77) M = Fe^{II} ; R = Et

(78) M = Fe^{II} ; R = H

it caused bilirubinuria and urobilinuria but also that the bile did not contain pigments with ethyl side-chains. The high toxicity of mesohaematin was demonstrated and it was argued that haematin would be unlikely to be a normal biological precursor of bilirubin.[54]

However, in more recent times the conversion of haematin into bilirubin has

† 'Haematins' exist as the dehydrated μ-oxo-dimer

[50] I. M. London, M. Yamasaki, and A. G. Sabella, *Fed. Proc.*, 1951, **10**, 217.

[51] H. Schöttmuller, *Münch. Med. Wochschr.*, 1914, **61**, 230.

[52] O. Schumm, *Z. physiol. Chem.*, 1916, **97**, 32.

[53] K. Bingold, *Z. Klin. Med.*, 1923, **97**, 257; *Folia. Haematol.* (*Leipzig*), 1930, **42**, 192; R. Duesberg, *Arch. Exp. Pathol. Pharmakol.*, 1934, **174**, 305; *Klin. Wochschr.*, 1938, **17**, 1353.

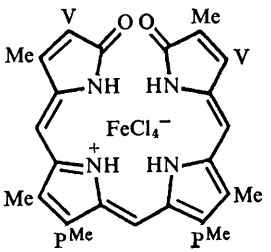
[54] Ref. 1 (*b*), p. 575.

been demonstrated both *in vivo*[55–58] and *in vitro*.[59] It was observed[55] that ^{15}N-labelled haematin is rapidly converted into faecal stercobilin (14) in dogs, and that when injected into rats with external bile fistulae, ^{14}C-labelled haematin is converted into bilirubin with an efficiency between 50 and 70%,[56] values which are comparable with those for haemoglobin itself.[57]

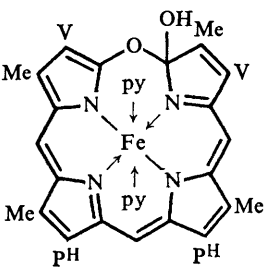
There are two modern theories of bile-pigment formation; one holds that the process involves non-enzymic coupled oxidation and hydrolysis whereas the second proposes an enzymic transformation.

Coupled Oxidation of Iron Porphyrins. In 1930, Warburg and Negelein[60] described the formation of a green compound on coupled oxidation of pyridine haemochrome (66) in presence of hydrazine, and the compound was called 'green haemin'. When treated with methanolic hydrogen chloride, 'green haemin' gave an ester which was subsequently shown[61] by Lemberg in 1935 to be biliverdin dimethyl ester ferrichloride (67), and only one isomer was isolated. Lemberg[62] also obtained crystalline biliverdin dimethyl ester (32) by coupled oxidation of haemoglobin in presence of ascorbic acid; because only one isomer was obtained it was widely believed that this pathway must be operative *in vivo*.

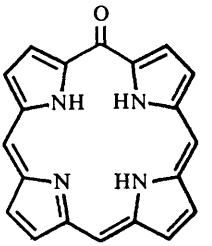
'Green haemin' was renamed pyridine verdohaemochrome by Lemberg, and a structure (68) was proposed for it.[63] A compound with an absorption maximum at 639 nm was shown to be formed before verdohaemochrome,[63] especially when the reaction was carried out with hydrogen peroxide and ascorbic acid. Removal of iron from this intermediate gave an oxyporphyrin; Lemberg favoured the



(67)



(68)

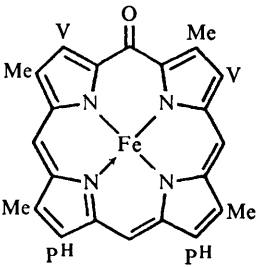


(69)

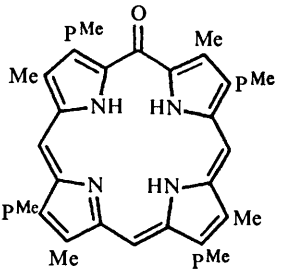
[55] I. M. London, *J. Biol. Chem.*, 1950, **184**, 373.
[56] A. L. Snyder and R. Schmid, *J. Lab. and Clin. Medicine*, 1965, **65**, 817.
[57] J. E. Ostrow, J. H. Handl, and R. Schmid, *J. Clin. Investigation*, 1962, **41**, 1628.
[58] H. Bénard, A. Gajas, M. Polonovski, and M. Tessier, *Compt. rend. Soc. Biol.*, 1946, **140**, 51; I. J. Pass, S. Schwarz, and C. J. Watson, *J. Clin. Investigation*, 1954, **24**, 283.
[59] J. E. Kench, C. Gardikas, and J. F. Wilkinson, *Biochem. J.*, 1950, **47**, 129; J. E. Kench, *ibid.*, 1954, **56**, 669.
[60] O. Warburg and E. Negelein, *Ber.*, 1930, **63**, 1816.
[61] R. Lemberg, *Biochem. J.*, 1935, **29**, 1322.
[62] R. Lemberg, J. W. Legge, and W. H. Lockwood, *Nature*, 1938, **142**, 148.
[63] R. Lemberg, B. Cortis-Jones, and M. Norrie, *Biochem. J.*, 1938, **32**, 171.

keto-tautomeric form (69) for oxyporphyrins and this has since been confirmed,[64] with the macrocycle being named 'oxophlorin'. The 639 nm compound was therefore characterized as the iron(III) complex (70) of the oxophlorin, and was found to be transformed into the corresponding verdohaemochrome by molecular oxygen.

Fischer and Libowitzky[65] also obtained an oxophlorin (71) from the oxidation



(70)



(71)

of iron(III) coproporphyrin-I. Treatment with oxygen in pyridine gave the corresponding verdohaemochrome, which was transformed into the biliverdin by treatment with alkali and then acid in 73% yield from verdohaemin.

When haemoglobin and ascorbic acid were exposed to air for 48 h, a compound with an absorption maximum at *ca.* 674 nm was obtained.[62] Treatment with sodium dithionite shifted the maximum to 629 nm. The chromophore was liberated from the 619 nm substance by treatment with ammonium sulphate followed by ethyl acetate in acetic acid. The freed material contained some haem, but biliverdin hydrochloride was formed with 1% hydrochloric acid. The substance with absorption maximum at 619 nm was termed 'choleglobin'[66] and was thought to be a bile pigment–iron complex; the 674 nm material appeared to be 'oxycholeglobin' because on evacuation and dialysis choleglobin was obtained.

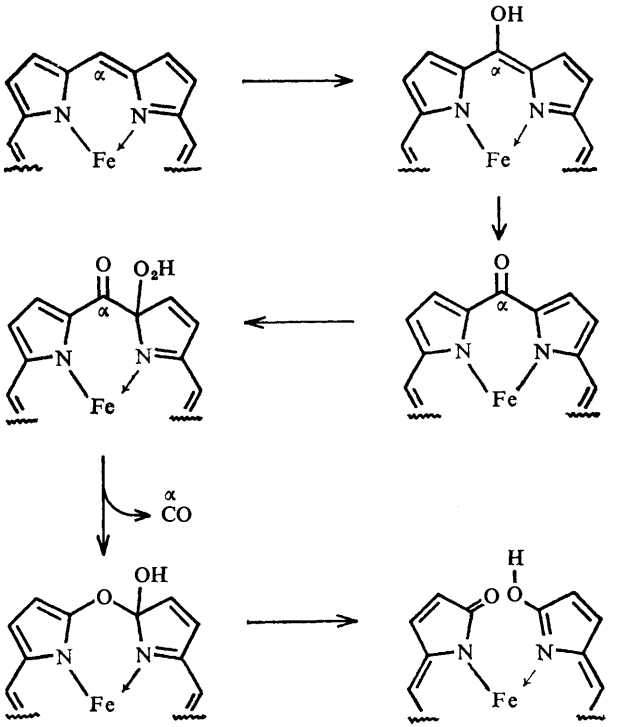
Choleglobin has never been obtained free from haemoglobin,[66] the reaction being prevented from going to completion by denaturation which occurs during its course. Nevertheless, choleglobin has been postulated[2] as an intermediate in bile-pigment formation as outlined in Scheme 12. The only difference between Schemes 11 and 12 is whether the protein and iron are removed before or after the oxidation, since it is now accepted that cholehaem is a mixture of compounds containing oxidized porphyrin rings. Lemberg *et al.*[67] have postulated a pathway

[64] A. H. Jackson, G. W. Kenner, and K. M. Smith, *J. Amer. Chem. Soc.*, 1966, **88**, 4539; *J. Chem. Soc.* (*C*), 1968, 302.
[65] H. Fischer and H. Libowitzky, *Z. physiol. Chem.*, 1938, **255**, 209; H. Libowitzky, *ibid.*, 1940, **265**, 191.
[66] R. Lemberg, J. W. Legge, and W. H. Lockwood, *Biochem. J.*, 1941, **35**, 328, 339, 363; R. Lemberg and J. W. Legge, *ibid.*, 1941, **35**, 353.
[67] E. C. Foulkes, R. Lemberg, and P. Purdom, *Proc. Roy. Soc.*, 1951, **B138**, 386.

Haemoglobin $\xrightarrow{\text{Oxidn.}}$ Choleglobin $\xrightarrow[-\text{protein}]{-\text{Fe}}$ Biliverdin $\xrightarrow{\text{redn.}}$ Bilirubin

Scheme 12

for the oxidative cleavage of the porphyrin nucleus, and this is shown in Scheme 13. The carbon atom which is lost during the course of the transformation is liberated in the form of carbon monoxide (*vide supra*).[4]

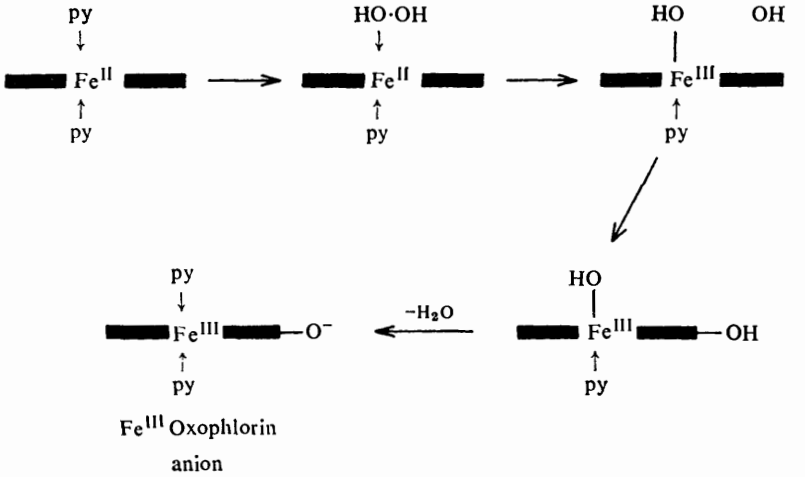


Scheme 13

Protoporphyrin-IX does not undergo ring-opening upon coupled oxidation with ascorbic acid,[59] and this seems to suggest some special role for the iron atom, apart from that already recognized for the transport of oxygen in haemoglobin. Bonnett and Dimsdale[68] allowed a series of metallo-octaethylporphyrins to react with hydrogen peroxide in pyridine; metallo-oxophlorins were only obtained in those cases (where the central metal ion was Fe^{II}, Co^{II}, Mn^{II}, and Mn^{III}) in which the metal atoms possessed an easily accessible higher oxidation state.

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

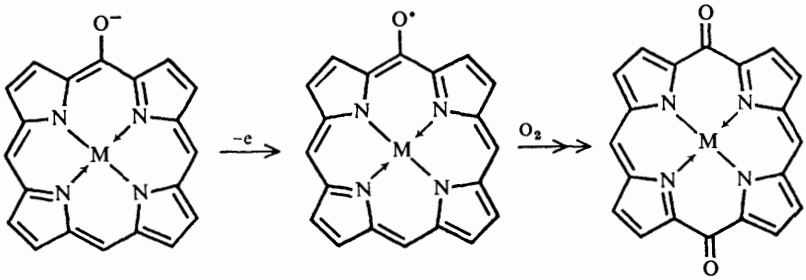
For example, in the iron(II) case, the product is an iron(III) oxophlorin, and since the *meso*-position and the metal ions are oxidized with peroxide, a mechanism (Scheme 14) similar to that occurring in aromatic hydroxylation with Fenton's



Scheme 14

reagent was proposed,[68] the great novelty being in the fact that the iron(II) species is situated within the porphyrin nucleus. This mechanism also accounts for the fact that a reducing agent is required to be present during the oxidation (*i.e.* coupled oxidation) of iron(III) porphyrins because the reaction as outlined (Scheme 14) is carried out on the iron(II) porphyrin.

Treatment of iron(III) oxophlorins with oxygen in pyridine affords high yields of verdohaemochrome, as mentioned earlier. However, treatment of other metallo-oxophlorins (central metal, *e.g.* Mg^{II} or Zn^{II}) with oxygen affords the corresponding metallo-dioxoporphodimethenes (72) *via* the anion (73) and



(73) (74) (72)

M = Mg, Zn

radical (74)[69] The tendency of iron(III) oxophlorins to react with oxygen by addition across the carbonyl group (see later) rather than at the opposite methine carbon may be associated with the fact that removal of an electron from the iron(III) oxophlorin anion [*cf.* (73)→(74)] might lead to the formation of an iron(IV) oxophlorin rather than an iron(III) oxophlorin radical; this possibility of electron loss from the metal rather than the ligand is supported by (i) the observation[70] that electrochemical one-electron oxidation of iron(III) porphyrins gives iron(IV) porphyrins and not the π-cation radical of the metalloporphyrin as in most other cases, and (ii), the implication[71] of iron(IV) porphyrins as intermediates in the functioning of haem systems such as peroxidase and catalase. It might be reasonable to expect an iron(IV) oxophlorin to react somewhat differently with oxygen than does a magnesium or zinc oxophlorin radical. It is also fascinating to consider that the facility of haems to be transformed into iron(III) oxophlorins is dependent upon the ability of the metal to be readily converted from the +2 to the +3 oxidation state[68] and that the further reaction of the oxophlorin with oxygen to give verdohaemochrome is linked with the ability of the metal atom to be transformed from the +3 into the +4 state. Combination of these factors with the facility of iron(II) porphyrins to bind oxygen reversibly *without* oxidation, and the co-ordination chemistry of high- and low-spin iron(II) which enables triggering of the co-operative effect,[72] makes iron a very special metal indeed and may serve to explain why Nature depends so greatly on a metal which is in itself so poisonous.

Though Lemberg claimed[1b] that oxidative rupture of haemin (75) led only to the -IXα isomer of biliverdin, it has since been established in several ways (see p. 370) that a random mixture of bile pigments actually results from the coupled oxidation. Table 3[14] clearly shows that there is little directive influence by the

Table 3 *Percentages of biliverdin isomers produced by coupled oxidation with ascorbic acid and/or hydrazine of protohaemin or deuterohaemin*[14]

Biliverdin isomer	*Protohaemin*		*Deuterohaemin*
	ascorbic acid %	*hydrazine* %	*hydrazine* %
α	33.0 ± 5.1	26.5 ± 3.0	14.8 ± 2.1
β (δ)	22.5 ± 6.1	19.6 ± 3.0	45.3 ± 3.1
γ	22.3 ± 4.0	20.5 ± 2.0	29.2 ± 2.0
δ (β)	21.9 ± 2.0	23.5 ± 6.0	10.7 ± 1.0

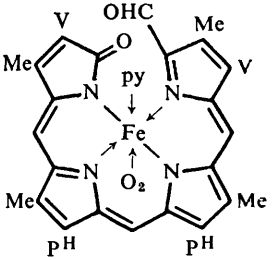
vinyl groups in haemin (75) and that in deuterohaemin (76) the β(δ)-isomer predominates.

A considerable degree of specificity has been noted in the coupled oxidation

[69] G. H. Barnett, B. Evans, and K. M. Smith, *Tetrahedron*, in press.
[70] R. H. Felton, G. S. Owen, D. Dolphin, and J. Fajer, *J. Amer. Chem. Soc.*, 1971, **93**, 6332.
[71] D. Dolphin and R. H. Felton, *Accounts Chem. Res.*, 1974, **7**, 26.
[72] M. F. Perutz, *Nature*, 1970, **228**, 726, 734; *New Scientist*, 1971, 676; W. Bolton and M. F. Perutz, *Nature*, 1970, **228**, 551.

of haemoproteins.[73] Myoglobin, when subjected to coupled oxidation, yields *only* the -IXα isomer, and haemoglobin affords both the -IXα and -IXβ isomers with no trace of the γ- or δ-isomers. Similar specificity was observed when the native haem was replaced with mesohaem (77) or deuterohaem (78).[73] This appears to suggest that the specificity of the rupture is a property of the haem binding rather than of the haem itself, and this is further confirmed by the fact that the cleavage becomes completely non-specific if the protein is denatured before coupled oxidation.[73] It is unlikely that the cleavage is made specific due to steric hindrance of the other *meso*-positions by the large protein molecule because *X*-ray studies have shown[74] that the α-carbon atom is in fact the *most* hindered, being situated at the bottom of the haem binding crevice.

The Enzymic Oxidation of Iron Porphyrins. In 1963, Nakajima *et al.*[75] reported the isolation of an oxygen-sensitive enzyme, 'haem α-methenyl oxygenase', from beef liver and guinea-pig liver. It was shown to catalyse the transformation of pyridine haemochrome (66) and haemoglobin–haptoglobin complex (haptoglobin is a haemoglobin-binding plasma protein) to a possible precursor of biliverdin, which was assigned structure (79) on inconclusive evidence.[76] The



(79)

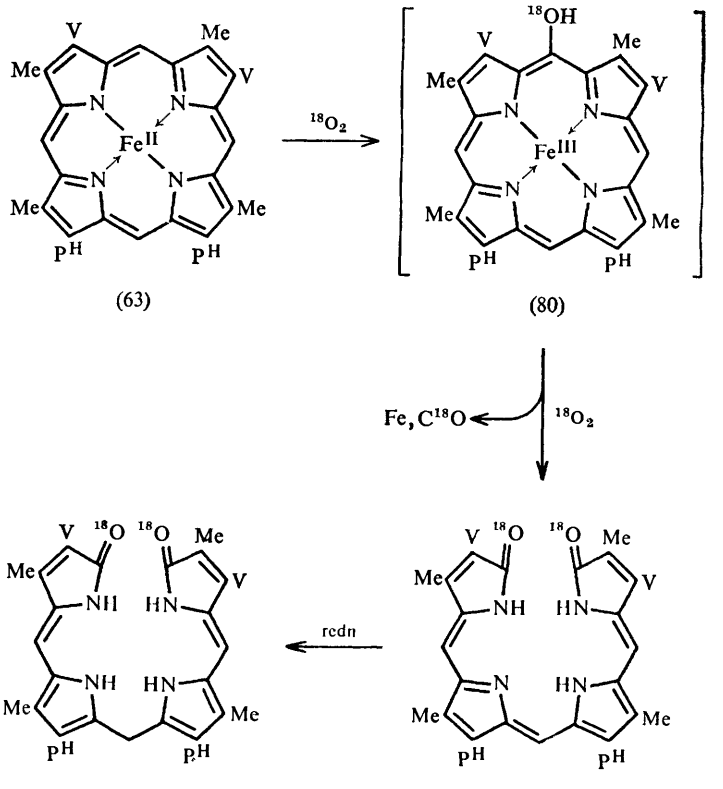
sites of highest enzyme activity were the liver and kidney, but curiously, it was totally absent from bone marrow and spleen, the two principal sites of haemoglobin breakdown. Later, Levin[77] confirmed that liver homogenates contained a substance which was active in cleaving pyridine haemochrome (66) but the active substance was only slightly heat-labile, was stable to extremes of pH, and was of low molecular weight, suggesting that it was not an enzyme at all. The active compound was shown to be a reducing agent and not to have a catalytic role; it was therefore suggested that the rapid disappearance of pyridine haemochrome was due to a non-enzymic coupled oxidation with endogenous reducing agents, and the proposed structure (79) of the product was discounted.[77] Murphy *et al.*[78] obtained the same results as Levin and they also showed that extracts from red

[73] P. O'Carra and E. Colleran, *F.E.B.S. Letters*, 1969, **5**, 295; P. O'Carra, in 'Porphyrins and Metalloporphyrins', ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 123.
[74] J. C. Kendrew, *Science*, 1963, **139**, 1259; M. F. Perutz, *Proc. Roy. Soc.*, 1969, **B173**, 113.
[75] H. Nakajima, T. Takemura, O. Nakajima, and K. Yamaoka, *J. Biol. Chem.*, 1963, **238**, 3748.
[76] H. Nakajima, *J. Biol. Chem.*, 1963, **238**, 3797.
[77] E. Y. Levin, *Biochemistry*, 1966, **5**, 2845; *Biochim. Biophys. Acta*, 1967, **136**, 155.
[78] R. F. Murphy, C. O'hEocha, and P. O'Carra, *Biochem. J.*, 1967, **104**, 6C.

algae contained a compound or compounds of low molecular weight which were active in cleaving porphyrins. The effect of the active factor was enhanced by NADPH; this was presumed to be due to regeneration of the active factor by reduction during the coupled oxidation.

Wise and Drabkin[79] have claimed that the haemophagous organ of dog placenta contains an enzyme system which catalyses the formation of biliverdin and carbon monoxide from haemin and haemoglobin. This system differed from that of Nakajima in two fundamental ways, (i) in substrate specificity, pyridine haemochrome being relatively ineffective, and (ii) in subcellular location, the activity being associated with the light mitochondrial fraction.

Kench *et al.*[80] noted that the splenic pulp of a haemolytic anaemia patient caused an enhancement in the production of bile-pigment precursors, normal splenic tissue being ineffective. Such enzymic activity was also observed in foetal liver, bone-marrow, and spleen, and the biliverdin-forming activity was found



Scheme 15

[79] C. D. Wise and D. L. Drabkin, *Fed. Proc..*, 1964, **23**, 223; *ibid.*, 1965, **24**, 222.
[80] J. E. Kench and S. N. Varma, *S. African Med. J.*, 1962, **36**, 794; J. E. Kench, F. E. duToit, and M. Green, *S. African J. Lab. and Clin. Medicine*, 1963, **9**, 273.

to be somewhat heat-labile, with bile-pigment yields considerably lower than reported by Nakajima. Indeed, Kench[81] and Schmid[82] also were unable to substantiate the work of Nakajima.

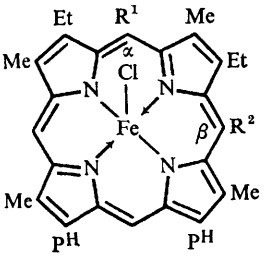
In 1970, Tenhunen *et al.*[83] published details of a microsomal enzyme system from liver which converts haem into bilirubin, and appears to be a mixed function oxidase. The system was called 'microsomal haem oxygenase' and requires molecular oxygen and NADPH as co-factors. When the enzymic reaction was carried out in an atmosphere of $^{18}O_2$, the resultant bilirubin (Scheme 15) possessed two ^{18}O atoms and an additional ^{18}O atom appeared in the expelled carbon monoxide. When carried out in a medium of $H_2{}^{18}O$, no label was incorporated, suggesting that no hydrolytic stage occurred between haem and bile pigment. The enzyme system is inhibited by carbon monoxide, and cytochrome P450 was implicated.

Scheme 15 proposes the intermediacy of an iron(III) oxophlorin (80); this possibility has been investigated by Kondo *et al.*[84] Tritiated α-oxymesoferrihaem (81) was injected into rats with bile fistulae and was found to be extensively converted into bile pigments, the expected mesobilirubin being identified. On the other hand, β-oxymesoferrihaem (82) was found to be a very poor precursor of bile pigments; the metal-free oxophlorins were also converted poorly into bile pigments. The authors concluded by analogy that α-oxyprotoferrihaem (80) is an intermediate in haemoglobin breakdown and that it undergoes further oxidation to bile pigment under the catalysis of an enzyme of definite specificity. This oxidation was postulated (Scheme 16) to proceed by addition of molecular oxygen across the oxophlorin carbonyl group followed by cheletropic fragmentation to give carbon monoxide and the biliverdin iron complex. Finally, the iron is removed to give biliverdin; the overall concepts in this scheme are in accord with the findings of Tenhunen *et al.*[83]

A NADPH-dependent microsomal haem cleavage system which does not seem to be associated with cytochrome P 450 has been reported by Yoshida *et al.*[85] Haem-cleavage activity had previously been reported[86] in chicken macrophages and this was similarly thought to be unassociated with P 450. Treatment of rats with $CoCl_2$ causes[87] stimulation of haem oxidation by hepatic microsomes; the rate of haem oxidation, however, has been shown to be unrelated to the microsomal content of P 450, and the conclusion has therefore been drawn that the microsomal enzyme system is not the same as that which metabolizes drugs, and therefore that cytochrome P 450 is not essential for haem oxidation.

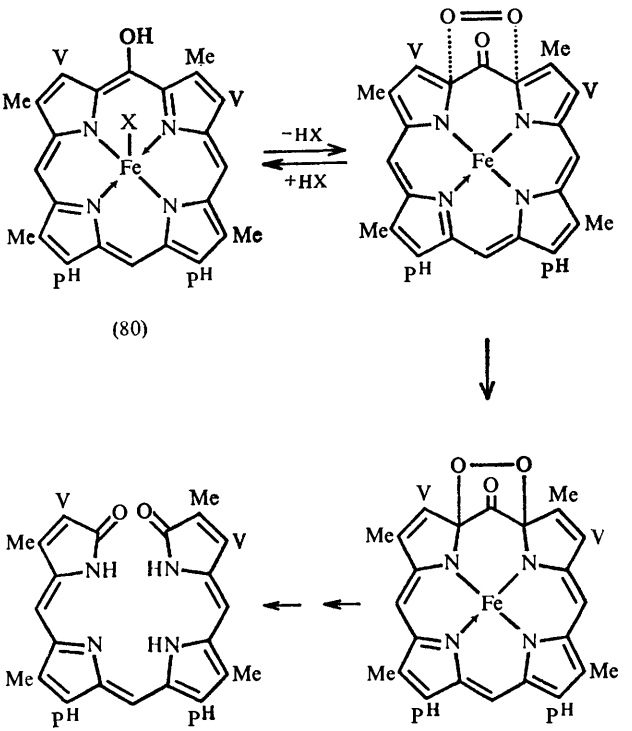
B. Algal Bile Pigments.—By comparison with the decades of experimentation which have been devoted to the investigation of catabolism of the blood pig-

[81] J. E. Kench, M. Green, and M. Hines, *S. African J. Lab. and Clin. Medicine*, 1964, **10**, 33.
[82] R. Schmid, *S. African J. Lab. and Clin. Medicine*, 1963, **9**, 276.
[83] R. Tenhunen, H. Marver, N. R. Pimstone, W. F. Trager, D. Y. Cooper, and R. Schmid, *Biochemistry*, 1972, **11**, 1716.
[84] T. Kondo, D. C. Nicholson, A. H. Jackson, and G. W. Kenner, *Biochem. J.*, 1971, **121**, 601.
[85] T. Yoshida, S. Takahashi, and G. Kikuchi, *J. Biochem.* (*Japan*), 1974, **75**, 1187.
[86] A. W. Nichol, *Biochim. Biophys. Acta*, 1970, **222**, 28.
[87] M. D. Maines and A. Kappas, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 4293.



(81) R^1=OH, R^2= H

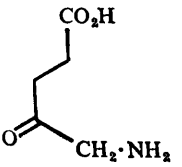
(82) R^1 = H, R^2= OH



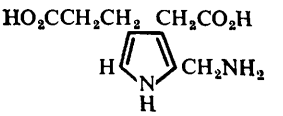
Scheme 16

ments, relatively little work has been carried out on the origin of the algal bile pigments. However, it is now clear that algal pigments are derived by oxidative rupture of porphyrin precursors, as might be expected from the 'type-IX' orientation of their substituents.

Illuminated cells of *Cyanidium caldarium* incorporated[88] δ-aminolaevulinic acid (83) (ALA) into phycocyanin, but in the dark, incubation of the alga with



(83)
ALA

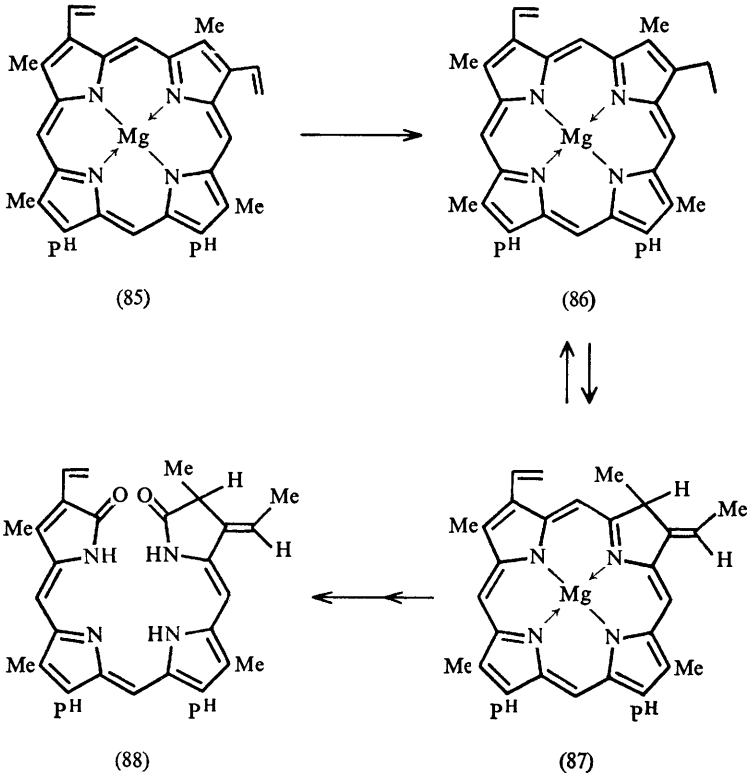


(84)
PBG

ALA resulted in excretion of porphobilinogen (84) (PBG), seven porphyrins, and a blue pigment, shown[89] to be the chromophore of phycocyanin. Consideration of the specific activities of products isolated after feeding isotopically labelled ALA allowed the conclusion[89] that the bile pigment was formed from ALA *via* PBG and a porphyrin, as in mammalian haem catabolism. However, the probable intermediates, uroporphyrinogen-III, coproporphyrinogen-III, protoporphyrin-IX, and possibly haem, have not been isolated[89] from *C. caldarium* cells actively synthesizing phycocyanin. During the synthesis of phycocyanobilin (the prosthetic group of phycocyanin), wild-type cells of *C. caldarium* produced equimolar amounts of carbon monoxide and phycocyanobilin,[90] suggesting that the CO and bile pigments are derived from the carbon skeleton of protoporphyrin-IX. These results were interpreted such that haem, [the iron(II) complex of protoporphyrin-IX], or a haemoprotein, should be the substrates for ring-opening to give the algal bile pigments.

In our view, it is at least as likely that the magnesium complex of protoporphyrin-IX is the metalloporphyrin intermediate in algal bile-pigment formation. Magnesium(II) protoporphyrin-IX (85) undergoes photochemical oxidation[91] to give compounds which have been formulated[92] as formylbiliverdins in a model series. The argument against this is that adapted cells of the blue-green alga *Tolypothrix tenuis* synthesize[93] phycocyanobilin in the dark, and therefore that, at least in this species, light is not an essential requirement. However, in the next section of this review, the novel ring-opening of metallochlorins to give[94] dihydrobiliverdin derivatives is discussed, and in this reaction the oxidative ring-opening occurs at a *meso*-position *adjacent* to the reduced ring. Scheme 17 outlines a possible route to phycocyanobilin from magnesium protoporphyrin-IX (85) in which the 4-vinyl is first reduced to ethyl to give (86); after a proto-

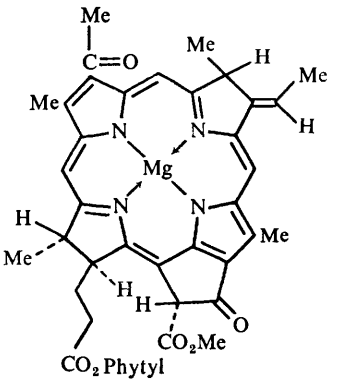
[88] R. F. Troxler and L. Bogorad, *Plant Physiol.*, 1966, **41**, 491; also, in 'Biochemistry of Chloroplasts' Vol. 2, ed. T. W. Goodwin, Academic Press, London, 1967, p. 421.
[89] R. F. Troxler and R. Lester, *Biochemistry*, 1967, **6**, 3840.
[90] R. F. Troxler, *Biochemistry*, 1972, **11**, 4235.
[91] J. Barrett, *Nature*, 1967, **215**, 733.
[92] J.-H. Fuhrhop and D. Mauzerall, *Photochem. and Photobiol.*, 1971, **13**, 453.
[93] Y. Fujita and A. Hattori, 'Studies on Microalgae and Photosynthetic Bacteria', University of Tokyo Press, Tokyo, 1963, pp. 431—440.
[94] J. A. S. Cavaleiro and K. M. Smith, *J.C.S. Perkin I*, 1973, 2149.



(85) (86) (88) (87)

Scheme 17

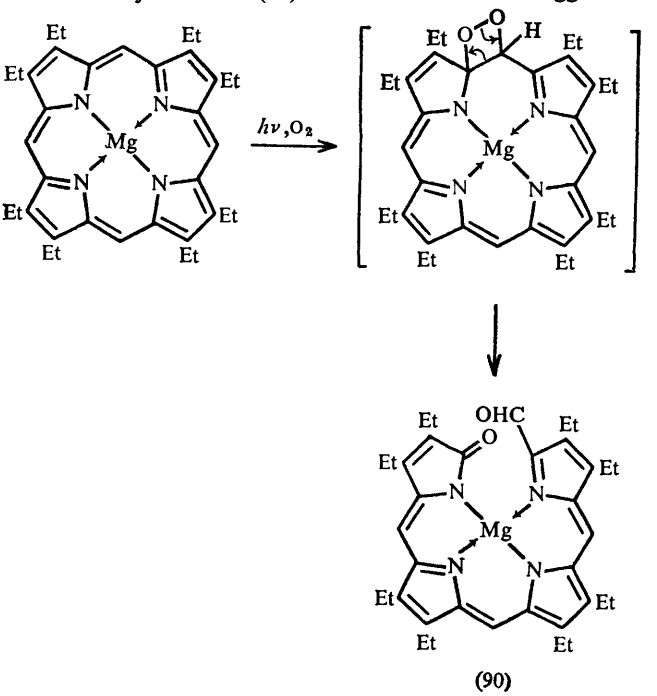
tropic shift to furnish the 'chlorin' (87), ring-opening could give (88), a possible precursor of the phycobilins. In normal chlorophyll-*a* biosynthesis, the 4-vinyl group present in magnesium protoporphyrin-IX is reduced to ethyl at a point



(89)

as yet uncertain, and the prototropic shift to give (87) from (86) is not unknown because bacteriochlorophyll-*b* has recently been assigned[95] the structure (89), the ethylidene group in ring B presumably being derived from an ethyl group *via* a proton shift.

C. Other Approaches to Bile Pigments from Porphyrins.—In 1938, Fischer and Bock showed[96] that photo-oxidation of the sodium complex of aetioporphyrin-I afforded, amongst other things, a bile pigment. Some time later, Barrett[91] showed that photo-oxidation of magnesium protoporphyrin-IX gave ring-opened compounds which were identified as biliviolins. The product from the same reaction in the magnesium octaethylporphyrin series was differently formulated as 1′-formylbiliverdin (90) and a mechanism was suggested[92] (Scheme

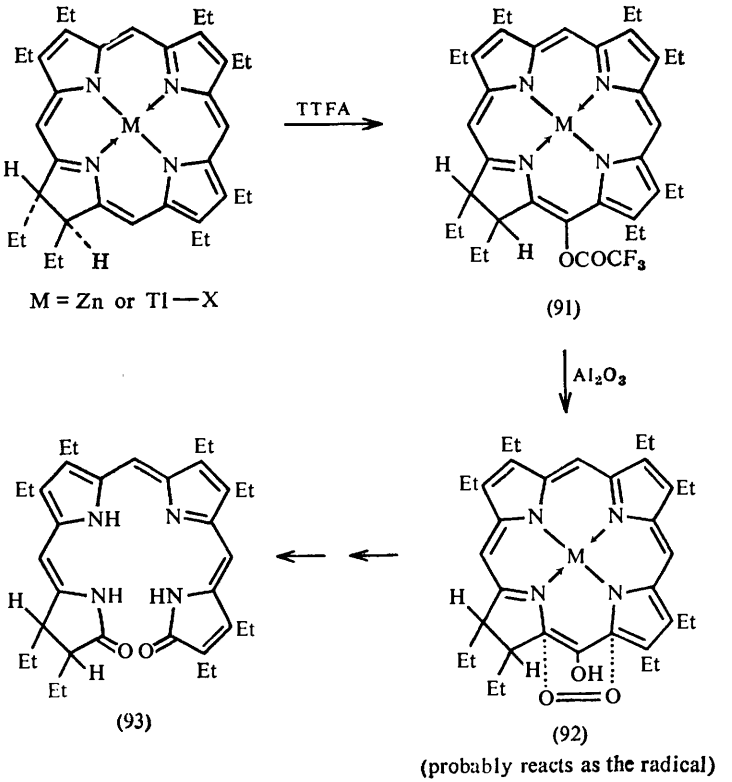


Scheme 18

18). In a similar type of reaction zinc chlorins give a mixture of the two possible formyl-dihydro-octaethylbiliverdins, in which the ring has been ruptured adjacent to the reduced sub-unit.[97]

[95] H. Scheer, W. A. Svec, B. T. Cope, M. H. Studier, R. G. Scott. and J. J. Katz. *J. Amer. Chem. Soc.*, 1974, **96**, 3714.
[96] H. Fischer and H. Bock, *Z. physiol. Chem.*, 1938, **251**, 1.
[97] P. K. W. Wasser and J.-H. Fuhrhop, *Ann. New York Acad. Sci.*, 1973, **206**, 533; J.-H. Fuhrhop, P. K. W. Wasser, J. Subramanian, and U. Schrader, *Annalen*, 1974, 1450.

Treatment of zinc(II) or thallium(III) chlorins with thallium(III) trifluoroacetate affords[94] the corresponding *meso*-trifluoroacetoxy-derivative (91) (Scheme 19),



Scheme 19

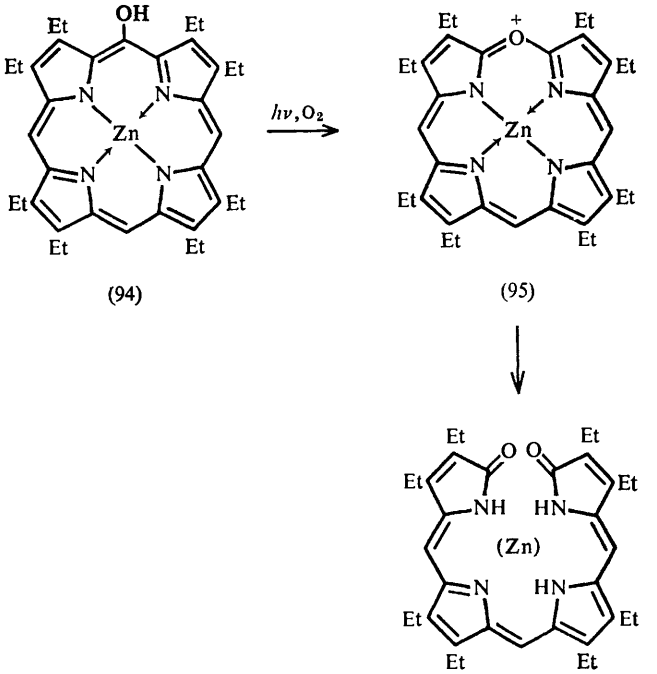
which can be hydrolysed to give the oxychlorin (92) (*cf.* the oxophlorin intermediate in haem catabolism); this compound (92) reacts spontaneously with oxygen to give the dihydro-biliverdin (93).

The most recent example of ring-rupture to be reported[98] is the photo-oxidation of zinc oxophlorins (94), which affords biliverdins *via* zinc oxaporphyrins (95) (Scheme 20).

5 Common Reactions of Bile Pigments

In this section we shall concentrate only upon those reactions which accomplish some kind of modification of the carbon skeleton of the bile pigment, thereby excluding discussion of reactions which might tend to be more characteristic of side-chains than of the pigment itself. On the other hand, we are also omitting

[98] S. Besecke and J.-H. Fuhrhop, *Angew. Chem.*, 1974, **86**, 125.



Scheme 20

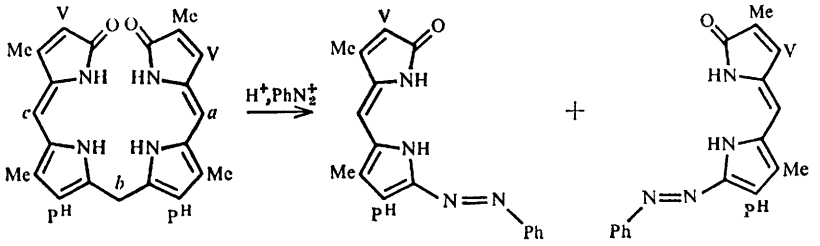
discussion of hydrogenation of the chromophoric group and complexation with metal ions, both of which are properties of the chromophore, but in which no change in the skeletal composition takes place.

A. Gmelin Reaction.—The first reaction of bile pigments to be investigated was the treatment of bile with nitric acid contaminated with nitrous acid, carried out by Tiedemann and Gmelin,[99] in 1826. This caused a series of colour changes from the orange-yellow of bilirubin, through green, blue, violet, red, and yellow. A great deal of work on this colour test for bile pigments was carried out by Siedel,[100] using both natural and more symmetrical model bile pigments. It is generally accepted that the first stage in the reaction is oxidation of bilirubin (yellow-orange) to give biliverdin (green); after this the absorption maximum gradually moves to shorter wavelength owing to interruption of the chromophoric pathway, firstly at a terminal inter-pyrrolic position, then at the other, and finally at the central methine carbon.[1b] Though the compounds presumed to give rise to these colours have in most cases been given names, their structural assignments are still confused.

[99] F. Tiedemann and L. Gmelin, 'Die Verdauung nach Versuchen', 1st edn., Karl Groos, Heidelberg and Leipzig, 1826.

[100] W. Siedel and W. Fröwis, *Angew. Chem.*, 1939, **52**, 38; *Z. physiol. Chem.*, 1941, **267**, 37; W. Siedel and E. Grams, *ibid.*, 1941, **267**, 49; W. Siedel, *Angew. Chem.*, 1943, **56**, 169.

B. Diazo Reaction.—The diazo reaction was discovered in 1883 by Ehrlich, who treated a solution of bilirubin in chloroform with diazobenzenesulphonic acid in acidified alcohol; a red colour was produced[101] which changed through violet to blue when treated with concentrated hydrochloric acid. [For a thorough treatment of this reaction see ref. 1(*d*), p. 59.] This colour test is positive with all bile pigments of the *a*,*c*-bilidiene type (6) which have a *b*-CH_2 group, but is negative, for example, with biliverdins. With unsymmetrically substituted bilirubins, the products are two azo-compounds (96) and (97) (Scheme 21). The



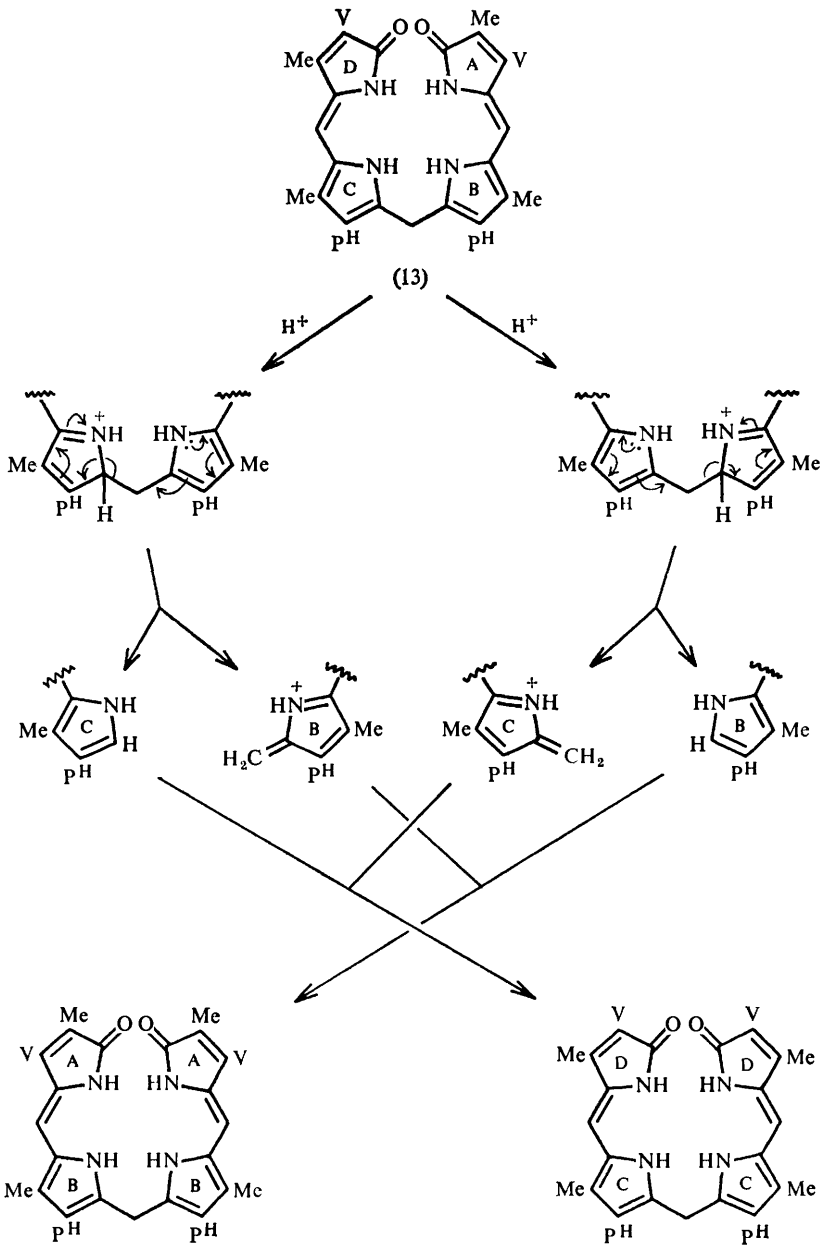
Scheme 21

mechanism of the diazo reaction has recently been investigated[102] and the central (*b*) carbon atom was shown to be liberated as formaldehyde.

C. Isomerization of Bilirubins in Acid Solution.—This reaction has a bearing also on the diazo reaction. Bilirubin has been shown[103,104] to undergo reversible acid-catalysed cleavage about the central methylene bridge, which leads to isomeric scrambling (Scheme 22) with production of bilirubins-IXα (13), -XIIIα (98), and -IIIα (99) in an equilibrium mixture. Under alkaline conditions, bilirubin does not isomerize. These findings have shown that reaction conditions employed using bilirubins must be carefully scrutinized or else one may be dealing with an isomeric mixture of pigments; *e.g.*, dehydrogenation of bilirubin with 1,4-benzoquinone in DMSO–acetic acid, or with ferric chloride in acetic acid, affords[103] a mixture of biliverdins.

D. Photo-oxidation of Bile Pigments.—Interest in the photo-oxidation of bilirubin has been stimulated by the observation[105] that hyperbilirubinemia in prematurely born infants can be treated by phototherapy, using sunlight or artificial visible light.[106] The process involves a self-sensitized reaction involving

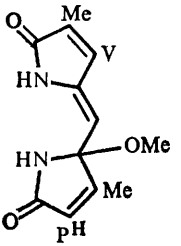
[101] P. Ehrlich, *Centr. Klin. Med.*, 1883, **4**, 721.
[102] D. W. Hutchinson, B. Johnson, and A. J. Knell, *Biochem. J.*, 1972, **127**, 907.
[103] R. Bonnett and A. F. McDonagh, *J.C.S. Chem. Comm.*, 1970, 238.
[104] A. F. McDonagh and F. Assisi, *J.C.S. Chem. Comm.*, 1972, 117; *Biochem. J.*, 1972, **129**, 797.
[105] R. J. Cremer, P. W. Perryman, and D. H. Richards, *Lancet*, 1958, **1**, 1094.
[106] J. Lucey, M. Ferreiro, and J. Hewitt, *Pediatrics*, 1968, **41**, 1047.



(98) (All arrows are reversible) (99)

Scheme 22

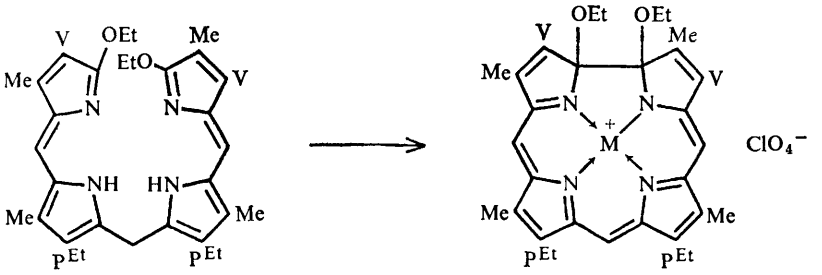
singlet oxygen.[107] The products isolated[108] from methanol solutions are methyl vinylmaleimide (36), haematinic acid (29), and various propentdyopent adducts [*e.g.* (100)]. Short irradiation times result in the production of biliverdin, but it



(100)

has been shown[109] that this pigment inhibits the sensitized and unsensitized photo-oxidation of bilirubin, and therefore biliverdin is probably not an intermediate in the main pathway of bilirubin photo-oxidation *in vitro*.

E. Cyclization of Bile Pigments to give Macrocycles.—Treatment of the bis-iminoether (101) from bilirubin diethyl ester with cobalt or nickel salts affords the cyclized tetradehydrocorrin salts (102).[110] The zinc biliverdin



(101) (102) M = Co or Ni

obtained[98] by photo-oxidation of zinc oxophlorins (94) can also be re-cyclized to the corresponding zinc oxaporphyrin (95) (Scheme 20).

[107] A. F. McDonagh, *Biochem. Biophys. Res. Comm.*, 1971, **44**, 1306.
[108] For reviews, see D. J. Ostrow, *Semin. Hematol.*, 1972, **9**, 113; D. A. Lightner, *Photochem. and Photobiol.*, 1974, **19**, 457.
[109] A. F. McDonagh, *Biochem. Biophys. Res. Comm.*, 1972, **48**, 408; D. A. Lightner, D. C. Crandall, S. Gertler, and Q. B. Quistad, *F.E.B.S. Letters*, 1973, **30**, 309.
[110] H. H. Inhoffen, H. Maschler, and A. Gossauer, *Annalen*, 1973, 141.