ON THE FORMATION OF BILE PIGMENTS FROM HEME PROTEINS

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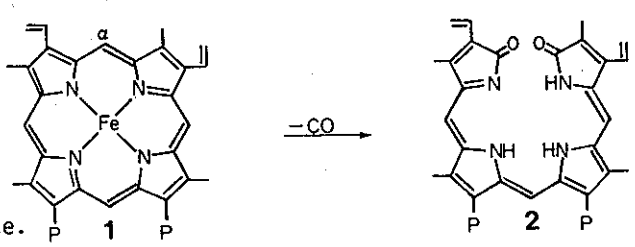
Dedicated to Professor R. B. Woodward on the occasion of his sixtieth birthday

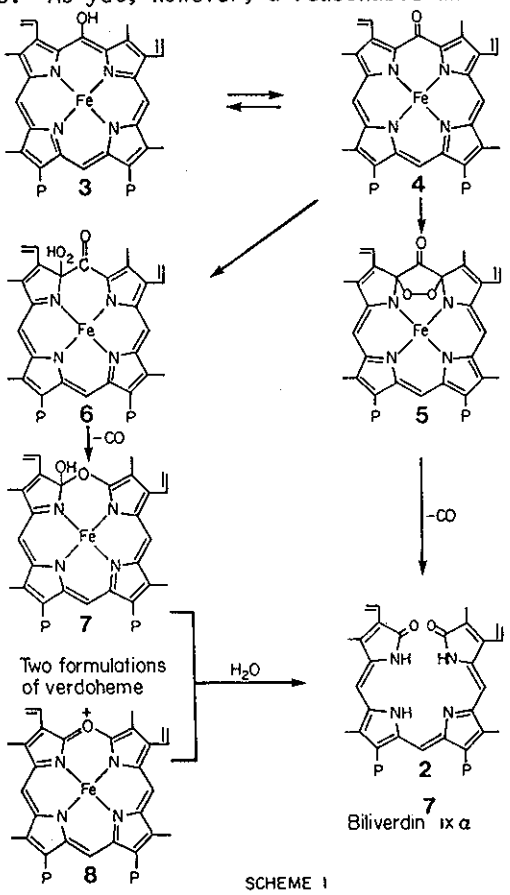
It is proposed that the catabolism of heme to biliverdin involves an initial epoxidation of a protein-bound porphyrin at the exposed γ-methine bridge. Rearrangement of the γ- to an α-epoxide followed by ring opening to a homooxaporphyrin would generate an electron-rich system capable of reacting with molecular oxygen to form a dioxetane. Fragmentation of the dioxetane to a mono formate ester of biliverdin followed by loss of carbon monoxide would give the bile pigment.

The *in vivo* degradation of heme to biliverdin presents an array of chemical transformations that, in many respects are without precedent in other chemical systems (1). Cleavage of the heme ring (**1**) has been shown to occur exclusively at the α-position accompanied by a remarkable decarbonylation to give biliverdin IXα (**2**) and carbon monoxide.



The evolution of carbon monoxide from blue-green alga (2) and plants (3) suggests that the formation of bile pigments in these systems proceeds in a similar fashion. The specificity of ring cleavage at the α-position of heme is absolute with the exception that biliverdin IXγ is found (4) in the integumental cells of the caterpillar of the cabbage butterfly *Pieris brassicae*. We feel that the natural occurrence of the γ-isomer is significant, and its occurrence must, along with that of the α-isomer, be explained as part of a general scheme for heme catabolism.

The reactions which account for bile pigment formation have been the center of a large number of studies. As yet, however, a reasonable and rational explanation of the total reaction mechanism has been elusive, resulting in a large and often contradicting literature (1,5). Tenhunen *et al* (6) have proven a requirement for molecular oxygen in the metabolic reaction. Using $^{18}O_2$ and $H_2{}^{18}O$ they showed that the terminal lactam oxygens of biliverdin, and the carbon monoxide oxygen, were all derived from molecular oxygen not water. The further observation (7) that tritiated Fe(III)α-oxomesoporphyrin (**4**) gave tritiated mesobiliverdin prompted the postulation that



SCHEME I

α-hydroxyheme (**3**) was an intermediate in the reaction. Jackson *et al* (8) then further suggested that addition of molecular oxygen to the tautomeric α-oxoheme (**4**) occurred to give the peroxide (**5**) which should spontaneously decarbonylate in a manner analogous to that exhibited (9) by tetraphenylcyclopentadienone.

Further evidence for the intermediacy of an α-hydroxyheme was provided by the early work of Lemberg (10) on the coupled oxidation of pyridine hemochrome (bis pyridine ferroprotoporphyrin). Treatment of pyridine hemochrome with ascorbate and oxygen resulted in formation of the green verdochrome (**7** or **8**) which could be readily hydrolysed with dilute HCl to biliverdin. Similarly oxygenation of a pyridine solution of mesohydroxyporphyrins resulted in the formation of green pigments. Lemberg (10) rationalised this in terms of a hydroperoxide attached on the α-pyrrolic carbon to give **6** which was followed by decarbonylation (Scheme 1). Lemberg extended this theory and suggested that the *in vivo* and coupled reactions took the same path.

Because of its metabolic significance the work on the coupled oxidation was extended by Bonnett and McDonagh (11) who showed that *in vitro* attack occurred randomly at all four methine bridges, discounting the earlier postulation of Lemberg (10) that the specificity of the metabolic reaction was due to an intrinsic activation of the heme α-position. However, specificity could be introduced into the coupled oxidation reaction when the heme was bound to globin or apomyoglobin. O'Carra (1b) determined the ratio of isomers formed on coupled oxidation of a variety of hemoproteins and found a greatly enhanced activity towards the α and, to a lesser extent, the β methine bridges. Thus it appears that the protein and not the porphyrin is imparting specificity to the α bridge. This

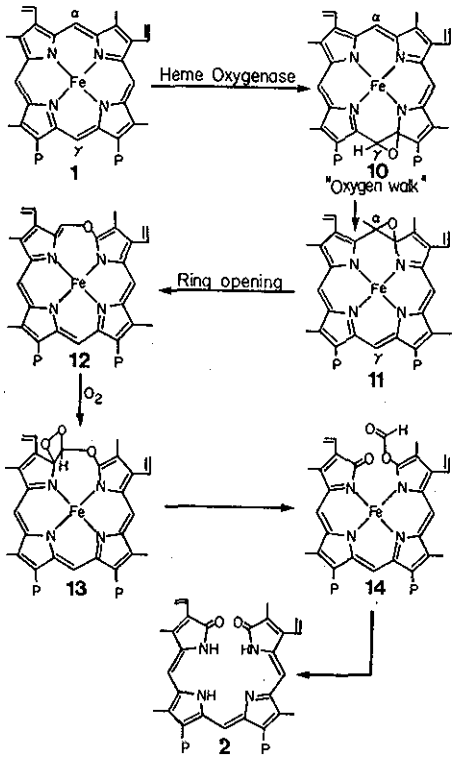
observation is particularly puzzling since in myoglobin (which showed 100% α specificity) the α methine bridge is buried deep within the protein (12) and thus apparently inaccessible to an attacking group. Brown (13) has suggested that the attacking oxygen molecule is bound to the central ferrous ion and that the protein orientates it to specifically attack the α position. There is, however, no example of oxygen bound to ferrous ion promoting oxidation of the porphyrin to which it is bound (14), nor to suppose that each of the separate proteins should have the same orientating effect.

Tenhunen *et al* (6) originally showed that both terminal oxygen atoms of bilirubin were derived from molecular oxygen. This apparently eliminates verdoheme (**7**, **8**) as an intermediate in the enzymic reaction since the conversion of verdoheme to biliverdin incurs a hydrolytic step whereby $H_2{}^{18}O$ should be incorporated into the bile pigment. Furthermore, the recent double labelling experiments of Brown *et al* (15) show that the two terminal oxygens of the bile pigment derive from *two different* oxygen molecules. This in turn eliminates symmetrically bridged intermediates of the type (**5**) which would fragment to give bile pigments where the terminal oxygen atoms were derived from the same molecule of oxygen.

It is thus apparent that none of the hypotheses concerning the chemical production of bile pigments from heme have stood the test of experiment. Moreover, none have convincingly explained the stereospecificity of the reaction. We propose here a mechanism (Scheme 2) involving the intermediacy of a porphyrin epoxide (**10**) and based upon chemistry with ample precedent which explains all of the diverse observations of both the *in vivo* and *in vitro* metabolism of heme.

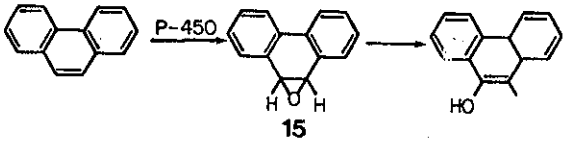
It had been suggested that the initial oxidant in the degradation of heme behaved like a modified cytochrome P-450 (1a, 16, 17). This view has

recently been challenged by Yoshida and Kikuchi (18) who suggest that heme oxygenase is a protein which by binding heme makes it more susceptible to oxidation. Thus it is not yet clear whether the *in vivo* reaction proceeds intermolecularly (6, 16) or intramolecularly (17) or what form the initial oxidation takes.



SCHEME 2

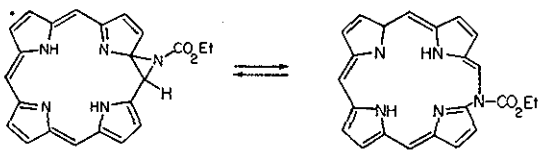
The mechanism of action of P-450 has oxene-like characteristics (19), and the P-450 induced hepatic detoxification of aromatic hydrocarbons (20) exhibits N.I.H. shifts (21) and proceeds through the intermediacy of arene oxides (**15**, Scheme 3). Hamilton (19) based his oxenoid mechanism on a comparison of the reaction shown in Scheme 3 to that of carbene (22) and nitrene (23) addition to aromatic nuclei which show similar three membered ring formation and ring opening reactions. Thus we propose that if heme degradation is initiated by enzymatic epoxidation of the porphyrin-periphery, attack will occur at the most exposed methine bridge to give an epoxide (**10**). Callot *et al* (24) and Grigg (25) have studied the reactions of porphyrins with carbenes

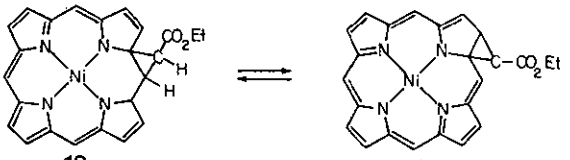


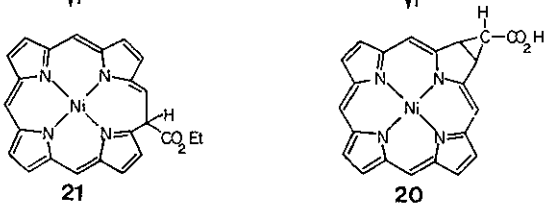
SCHEME 3

and nitrenes, where in both cases three membered ring systems (**16** and **18**) analogous to the epoxide (**10**), are readily prepared.

Callot *et al* (26) also observed a series of facile rearrangements of the homoporphyrin (**18**) by means of successive carbon-carbon bond migrations around the periphery to give compounds of the type **19** and **20**. Similar rearrangements of arene oxides are well documented and have been the subject of much study (27). "Oxygen walk" reactions (eg. **22** → **23**) can be induced both thermally (26) and photochemically (29) and seem to be a common feature of aromatic epoxides. The facile rearrangement of the homoporphyrins and arene epoxides suggest that the analogous "oxygen walk" of a γ-epoxide (**10**) to an α-epoxide (**11**) could occur.

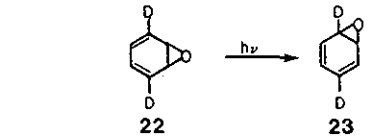




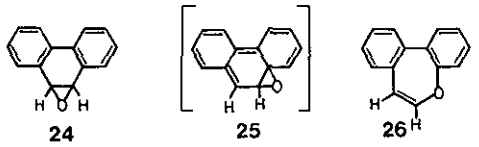


Thus in the coupled oxidation of hemoglobin and myoglobin, epoxidation at the γ-methine bridge followed by rearrangement to and further reaction at the α-position would account for the observed stereospecificity of "attack" at the least accessible α-position. Since the mode of binding of heme to heme oxygenase is unknown it is not yet clear whether direct attack at the α-methine bridge occurs, or if a rearrangement to the α-position occurs after attack at another position.

What further reaction could the epoxide **11** undergo and why the α rather than the γ-isomer? 9,10-Didehydro-9,10-epoxyphenanthrene (**24**) undergoes a photochemically induced "oxygen walk" to the isomeric compound **25** which in turn tautomerises to 2,3:4,5-dibenzoxepin (**26**) (29). Such tautomerisations are well known for arene oxides and studies (30) on the benzene oxide (**27**) ⇌ oxepin (**28**) equilibrium have shown it to be both sensitive to the nature of the substituents R_1, R_2, and to solvent effects (31). Thus when R_1=R_2=H the epoxide is favoured whereas when R_1=R_2=CH_3 the equilibrium almost completely favours the oxepin (**28**). As expected when R_1=H; R_2=CH_3 approximately equal amounts of each tautomer are present. Moreover the epoxide form is stabilised by polar solvents and the oxepin form by non polar solvents. Heme epoxidation at a γ-methine bridge followed by an "oxygen walk" to the α-position would then, as a result of the non polar environment around the α-methine bridge, favour opening of the epoxide **11** to the homooxaporphyrin derivative **12**. Completely analogous ring openings have been observed for the *meso*-homoporphyrin (**18** → **21**) (24) and the *meso*-homoazaporphyrin (**16** → **17**) (25). So far then oxidation of the more exposed γ-position, an "oxygen walk" to the α-position, followed by a ring opening in the non polar environment around the α-methine bridge could

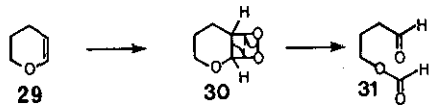


22 **23**



24 **25** **26**

27 **28**



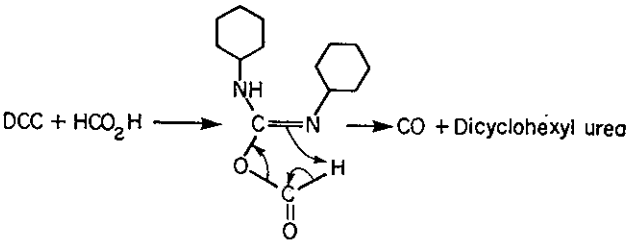
29 **30** **31**

lead to a ring expanded porphyrin (**10**→**11**→**12**). This sequence of events offers an explanation for the stereospecificity of heme degradation as well as the occurrence of biliverdin IXγ from the cabbage butterfly, but what could be the metabolic fate of the ring expanded porphyrin **12**?

Compound **12**, an α-homooxoheme contains an especially electron rich olefinic double bond which is both an enol ether and enamine. Electron rich double bonds react with molecular oxygen to give dioxetanes, thus the vinyl ether (**29**) gives, with singlet oxygen, the compound **30** (32). Not surprisingly Jackson *et al* (33) have shown that singlet oxygen is not involved in heme catabolism. However, as a double bond becomes very electron rich it will react with a ground state triplet oxygen. Thus tetra(dimethylamino)ethylene gives a dioxetane which fragments to give two moles of tetramethyl urea (34). Fragmentation to dicarbonyl compounds is a characteristic reaction of dioxetanes, and the opening of **30** → **31** is typical (32). Returning to the ring expanded porphyrin one can see that reaction with molecular oxygen would give the dioxetane (**12**→**13**) and that ring opening of the dioxetane would give the bile pigment **14**. The tetrapyrrole **14** is in fact biliverdin in which one of the terminal lactams is constrained in an enolic form as a formate ester.

We have now arrived at a stage where carbon monoxide can be liberated. Derivatives of formic acid in which the hydroxyl group of the carboxylic acid has been replaced by a good leaving group readily decompose to give carbon monoxide. The best known example of this is the spontaneous conversion of formyl chloride to carbon monoxide and HCl. Even formic acid itself when heated gives CO and water (35). Formic anhydride may be

prepared from dicyclohexyl carbodiimide (DCC) and the anhydride rapidly decomposes above 0° to formic acid and carbon monoxide (36). When formic acid is added to an excess of DCC at -78° no CO is evolved, but as the solution is warmed carbon monoxide is evolved and dicyclohexyl urea precipitated (37). From the stoichiometry of the reaction we have shown that it proceeds in the manner outlined in Scheme 4. This decomposition mimics that we propose for the formation of biliverdin (**2**) and carbon monoxide from **14**. Hence the mechanisms for heme catabolism outlined in Scheme 2 account not only for the specificity of the reaction, but also involve chemistry and intermediates for which there are ample precedent. Moreover it is apparent that while both terminal lactam oxygens of the bile pigment are derived from molecular oxygen the mechanism in Scheme 2 requires that it be derived from two different oxygen molecules. This is consistent with Brown's (15) labelling experiments which show a two-molecule mechanism is operative.

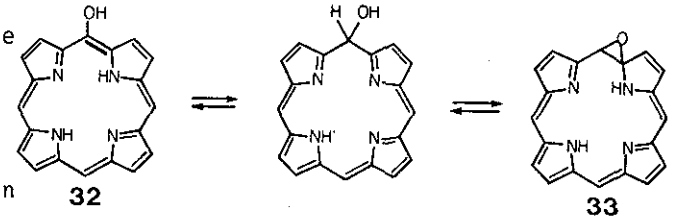


SCHEME 4

The formation of carbon monoxide during both *in vivo* formation of bile pigments and in the coupled oxidation of pyridine hemochrome suggests that these reactions have much in common. Indeed *in vivo* conversion of α-hydroxyheme (**3**) to biliverdin (**2**) led to the postulation (7) that **3** is a common intermediate in both reactions. So far we have not invoked the intermediary of a *meso*-hydroxyporphyrin. However, if heme oxygenase does

not function as a modified cytochrome P-450 then the hydroxylation of the bound heme in the presence of NADPH and cytochrome reductase could well initiate heme catabolism via a *meso*-hydroxyporphyrin. Nevertheless, one must note that a *meso*-hydroxyporphyrin (**32**) and porphyrin epoxide (**33**) are tautomeric (Scheme 5), such that invoking one could automatically include the other.

Finally, on the basis of spectral data, Dimsdale (38) has proposed that chromatography on alumina of a *meso*-hydroxyporphyrin gave the epoxide and hemo-oxaporphyrin corresponding to (**11**) and (**12**).



32 **33**

SCHEME 5

CONCLUSIONS

We propose here a mechanism to account for the catabolism of heme to biliverdin and carbon monoxide. This mechanism is consistent with, and analogous to, chemistry already reported for porphyrins and metalloporphyrins. Moreover the present mechanism also accounts for the two oxygen-molecule reaction which is required as a result of recent *in vivo* labelling studies (15).

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