HAEM CATABOLISM AND COUPLED OXIDATION OF HAEMPROTEINS*

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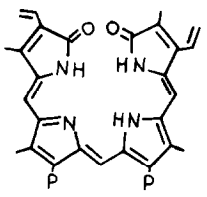
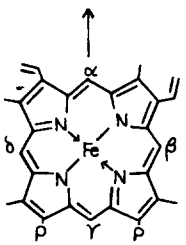
1. Introduction

In vertebrates, protohaem is catabolised via biliverdin to bilirubin. Since the bilirubin formed is almost entirely the IX α isomer [1] it is generally accepted that the initial step of haem catabolism involves cleavage specifically at the α-methine bridge to yield biliverdin IXα. (See scheme 1; the IXβ, IXγ and IXδ isomers of biliverdin would be produced respectively by cleavage at the β, γ and δ-methine bridges).

The mechanism of the specific *in vivo* cleavage is a controversial issue of long standing. Lemberg [2,3] many years ago advocated a mechanism similar to the non-enzymatic *in vitro* coupled oxidations of pyridine haemochrome and haemproteins with ascorbate, the products of which may be readily converted to biliverdin. However, this idea fell into disfavour with the demonstration that the coupled oxidation of pyridine haemochrome involves random cleavage of the methine bridges rather than specific cleavage at the α bridge [4,5]. Workers in the field have since concentrated on the search for an enzyme system which would specifically cleave heam or some derivative of it at the α-methine bridge. The problem seemed to have been solved by the work of Nakajima and coworkers, who in an extensive series of papers (e.g. Refs. 6–9) described a soluble hepatic enzyme "haem α-methenyl oxygenase" which was claimed to catalyse the cleavage of pyridine haemochrome specifically at the α bridge to yield a precursor of biliverdin IXα.

However, our investigations, after exhaustive rechecking, [10,11,12] have convinced us that the haemochrome-cleaving activity described by Nakajima et al. is entirely non-enzymatic and due to coupled oxidation of the haemochrome with endogenous ascorbate. This reaction seems to be similar in all respects to the *in vitro* system described many years previously. Other workers have also reported their inability to confirm the conclusions of Nakajima et al. [13,14,15]. Using our chromatographic technique of isomer analysis described in table 1, which is far more sensitive and discriminating than the degradation technique used by Nakajima [8,9], we find that the product formed from pyridine haemochrome by extracts from liver and other tissues contains all four isomers, and only 30–33% of the α isomer [11]. The *in vitro* coupled oxidation of pyridine haemochrome with ascorbate produces an identical mixture of isomers (table 1). This lack of specificity seems to rule out any physiological role for this system.





Scheme 1. (P = $-CH_2-CH_2-COOH$).

* First paper in a series on Bilin Metabolism.

Table 1

Isomeric composition of biliverdin products. The biliverdin preparation is methylated (in 1.5 ml 5% methanolic H_2SO_4, 16 h, 2°, under N_2), transferred to chloroform, washed free of acid and evaporated to dryness. It is then applied to a Silica Gel G t.l.c. plate and double-developed (in one dimension) with n-heptane-ethyl methyl ketone-acetic acid (10:5:1). The isomers, which separate in the order (front to back) $\gamma, \beta, \delta, \alpha$ [12], are scraped off, eluted into acetone and the relative proportions are estimated spectrophotometrically at 650 nm.

Haem derivative incubated aerobically with ascorbate*	% Isomer composition of biliverdin product			
	IXα	IXβ	IXγ	IXδ
Pyridine haemochrome	33	20	24	23
Myoglobin	100	0	0	0
Myoglobin in 8 M urea	32	21	25	22
Haemoglobin	80-65	20-35	0	0

* Pyridine haemochrome: incubated and product isolated as described by Murphy et al. [10]. Haemproteins: 50 mg. myoglobin (sperm whale or horse heart metmyoglobin, crystalline) or 50 mg, haemoglobin (bovine methaemoglobin X 2 cryst. or fresh oxyhaemoglobin preparations) in 10 ml. 0.1 M pH 7 phosphate buffer were incubated aerobically with 10 mg. ascorbate at 37° for 2 hours in 50 ml. beakers. The incubation mixtures were then cooled in an ice bath, 3 ml. acetic acid and 8 ml. 5 N HCl added, and the remaining heamin extracted quickly into peroxide-free ether (15 X 2). The biliverdin was then extracted quickly into chloroform (5 ml), and evaporated to dryness under a stream of nitrogen.

Heamin itself does not undergo any appreciable coupled oxidation with reductants under physiological conditions and seemed a better and more natural substrate than pyridine haemochrome. Many tissue extracts did in fact cleave haemin to biliverdin. However, we found that in general the products formed were again non-physiological mixtures of isomers. The activity seemed to be due to random haemochrome formation between the haemin and exposed amino or imidazolyl groups on any proteins in the extracts, followed by coupled oxidation of the haemochromes with reductants such as ascorbate.

We therefore re-investigated the direct, non-enzymatic breakdown of haemproteins by coupled oxidation with ascorbate which can be carried out under physiological conditions of temperature, pH and oxygen tension [3,13]. Biliverdin is produced directly, but in haemproteins containing thiol groups (e.g. haemoglobin) the latter tend to react with the very reactive vinyl side-chains of the biliverdin leaving a variable proportion of the pigment attached through artefact thio-ether bonds [3]. We have minimized this interaction by isolating the biliverdin formed as rapidly as possible (cf. table 1) but we still lose quite a proportion (30–50% in the case of bovine haemoglobin). In equating the isomeric composition of the isolated biliverdin with the relative composition of the total biliverdin formed, we make the assumption that the four isomers interact approximately equally with the thiol groups as is indicated by control incubations of the isomers with thiol-containing proteins.

This problem did not arise with myoglobin, which contains no thiol groups, and we obtained the most clear-cut results with this haemprotein. The biliverdin formed was entirely the IXα isomer. When the reaction carried out under denaturing conditions (in 8 M urea) the specificity was lost and a random mixture of all four isomers was obtained (table 1).

The result was unexpected since the X-ray diffraction work of Kendrew et al. [17] has shown that the α bridge of the haem group of myoglobin is situated at the bottom of the haem-containing crevice and is surrounded by hydrophobic amino acid side-chains. The γ bridge on the other hand is quite exposed and the β and δ bridges are also quite accessible from the exterior. The specific cleavage at the α-methine bridge must therefore be a positive effect of the haem-binding site rather than a masking of the other three bridges.

The biliverdin obtained by coupled oxidation of haemoglobin was also predominantly the IXα isomer but always with a proportion of the IXβ isomer which varied from 20% to 35% with different haemoglobin preparations and under different conditions. No traces of the IXγ or IXδ isomers were formed (table 1). Therefore a definite specific labilization is also apparent here though the specificity is not as absolute as in myoglobin.

The 2.8 Å-resolution model of haemoglobin constructed by Perutz et al. [18] indicates that the β-methine bridge of haem is in a more hydrophobic environment in all four heam-binding sites of haemoglobin than in myoglobin. Hydrophobic environments may therefore be the basis of the specific cleavage. Alternatively, conformational interactions of the subunits leading to perturbations of the haem crevice during the coupled oxidation may be responsible for the production of the IXβ isomer. We find that catalase (another tetrameric haemprotein) on coupled oxidation also yields specifically the IXα and IXβ isomers of biliverdin.

Preliminary results indicate that a microsomal haemprotein (problably cytochrome P450 which, like myoglobin and haemoglobin, contains protohaem and interacts with oxygen) also undergoes coupled oxidation in the presence of NADPH and ascorbate with the production of biliverdin IXα.

In view of these results it now seems unnecessary to retain the conventional concept of a haem-cleaving enzyme system since the haemproteins themselves seem to to have all the attributes being sought in such an enzyme system.

The production of significant proportion of biliverdin IXβ from haemoglobin may seem to conflict with the evidence that the bilirubin produced *in vivo* is almost entirely the IXα isomer, particularly since haemoglobin breakdown contributes the bulk of this bilirubin. However the yield of bilirubin from *in vivo* haemoglobin breakdown is not quantitative, varying between 60 to 80 per cent (cf. ref. 19). The 20 to 40 per cent of the haemoglobin haem not accounted for as bilirubin is thought to be catabolized by another route [19]. This 20 to 40% discrepancy corresponds closely with the 20 to 35% of biliverdin IXβ formed in our *in vitro* coupled oxidations of haemoglobin. Furthermore we have found that biliverdin reductase (the enzyme system which converts biliverdin to bilirubin) reduces biliverdin IXβ (and also IXγ and IXδ) at an extremely slow rate compared to biliverdin IXα (Colleran and O'Carra, unpublished). It is probable therefore that very little, if any, biliverdin IXβ formed *in vivo* would appear as bilirubin. The production of such a proportion of biliverdin IXβ from haemoglobin may therefore take place *in vivo* also.

Some further results suggest an alternative explanation: When coupled oxidation of small quantities of

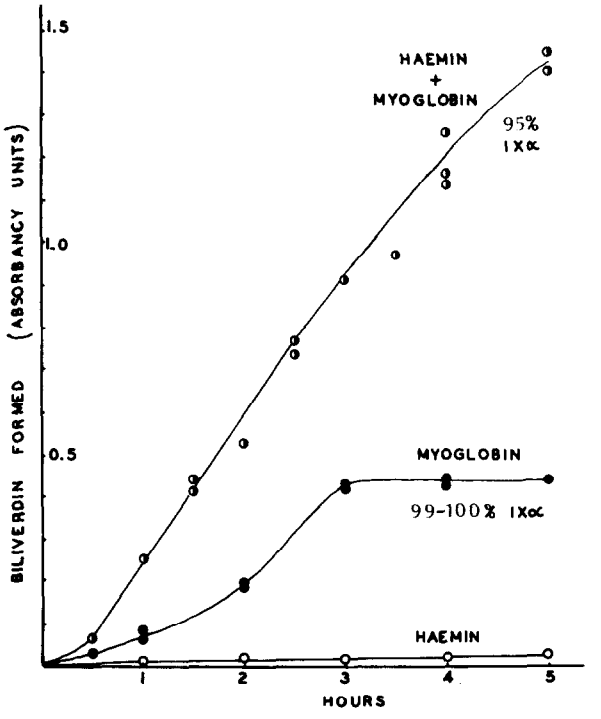


Fig. 1. (apo)Myoglobin as an α-methine-specific haem-cleaving enzyme. Aliquots of 0.92 μmoles haemin (o); 0.15 μmoles metmyoglobin (•); or 0.92 μmoles haemin + 0.15 μmoles metmyoglobin (⊙) were incubated aerobically in 50 ml. beakers with 10 mg. ascorbate in 6 ml. 0.1M phosphate buffer pH 7 for the indicated periods of time. (A separate incubation mixture was set up for each time point; the haemin was added as 1 ml. of a solution made by dissolving 6 mg. of haemin in 0.1 ml. of 5% sodium carbonate and diluting to 10 ml. with 0.1M phosphate buffer pH 7). The biliverdin product was isolated as described in table 1, the chloroform extract evaporated under nitrogen to c. 0.5 ml., made up to 2 ml. with chloroform and the absorbancy at 650 nm measured. These values were plotted directly as absorbancy units vs. time of incubation. The biliverdin aliquots were subsequently analysed for their isomer composition as described in table 1. The biliverdin produced from myoglobin is almost entirely IXα, that from haemin + myoglobin is over 95% IXα, while the trace of biliverdin produced from haemin alone is a random mixture. The lag phase in the curves appears to be caused by the necessary conversion of most of the metmyoglobin to oxymyoglobin. The levelling-out of the curve for myoglobin alone after 3 to 4 hours corresponds with essentially complete disappearance of its haem complement.

myoglobin or haemoglobin was carried out in the presence of an excess of added free haemin, the yield of biliverdin far exceeded the original haem content of the haemoprotein and the specificity of cleavage was retained (α bridge only in myoglobin; α and β in haemoglobin). A typical result obtained with myoglobin is illustrated in fig. 1.

Since haemin itself is not degraded at a significant rate under the conditions used (fig. 1), these results indicate that the apo-haemproteins cleave the added free haemin with both the specificity and turn-over characteristic of enzymes, the haem-binding sites being equivalent to active sites.

Exchange of haem between haemoglobin and the microsomal cytochrome *P*450 (via haemopexin) has been demonstrated [20,21] and methaemoglobin loses its haem readily to apomyoglobin *in vitro* [22]. It is therefore possible that the haem of haemoglobin, after rupture of the senescent erythrocyte, could be cleaved entirely to biliverdin IXα by a repetitious cycle of breakdown in one of the absolutely α-specific haemproteins, with continuous regeneration of this haemprotein by transfer of haem from haemoglobin. The breakdown of intraveneously injected free haemin [23] could also be accounted for by such a mechanism. The recent description [24] of the conversion of haemin to bilirubin by a microsomal system may be explained by a similair process, probably involving cytochrome *P*450 as postulated above, with a subsequent reduction of the biliverdin to bilirubin by biliverdin reductase in the extracts. Note that in such a mechanism we do not envisage cytochrome *P*450 acting in its usual role as a hydroxylase, but rather the apo-protein acting as an α-methine-specific haem oxidase.

In summary, our results lend strong support to a somewhat modified version of the long-standing hypothesis of Lemberg that coupled oxidation of haemproteins is the basis of the mechanism of *in vivo* haem breakdown.

Acknowledgements

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