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Haemoglobin Catabolism: the Role of Ferrihaems in Studies of the Degradation Pathway

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The importance of ferrihaem aggregation in studies of haemoglobin catabolism is assessed in the light of recent work. Experimental evidence is put forward suggesting that monomeric ferrihaems are degraded much more readily than dimeric species. This may offer an alternative explanation for the apparent 'apoprotein catalysis' recently observed.

Several investigations of the mechanism of haemoglobin catabolism have involved the use of protoferrihaem (synonyms: haemin, haematin) as haem source (Gray *et al.*, 1972; O'Carra & Colleran, 1969; Colleran & O'Carra, 1970; Murphy *et al.*, 1967; Tenhunen *et al.*, 1972; Lathe, 1972). In these studies little account has been taken of aggregation phenomena, the existence of which is now well established in haem systems (Falk, 1964). Recent work (Brown *et al.*, 1970*a*; Jones *et al.*, 1973*a*) has confirmed that, in aqueous solution, ferrihaems are dimerized according to the equation:

$$2M \overset{K}{\rightleftharpoons} D + H^+$$

where M and D refer to monomeric and dimeric ferrihaem respectively, and $K = [D][H^+]/[M]^2$. The value of K (the pH-independent dimerization constant) depends on a number of factors, including the nature of the porphyrin side chains. Changes between monomeric and dimeric species may be conveniently monitored spectroscopically, since the two forms of ferrihaem have quite different spectra in the 400nm region (see Fig. 2). The structural nature of ferrihaem dimers is only partially understood, but is known to involve an oxo bridge (Fe–O–Fe) between the component haem molecules (Brown *et al.*, 1969).

Of particular significance has been the finding of a marked, and somewhat unexpected, difference in degree of dimerization between protoferrihaem ($K = 4.5$) and deuteroferrihaem ($K = 1.9 \times 10^{-2}$), these compounds differing only in the nature of the porphyrin side chains. Thus at a concentration of 100μM (pH7, sodium–potassium phosphate buffer) protoferrihaem contains about 1% monomer whereas the corresponding value for deuteroferrihaem is 15%. Differences in overall reactivity between deuteroferrihaem and protoferrihaem may reflect this difference in state of aggregation rather than the specific nature of the side chains. This has indeed been shown to be the case in studies of the catalase activity of these compounds (Brown *et al.*, 1970*b*) and of their degradation by H_2O_2 (Jones *et al.*, 1973*b*).

It is important that results obtained from any system where ferrihaem is used should be carefully assessed in the light of this recent work, especially where substances are present which may alter the monomer–dimer equilibrium. Such substances include ligands which bind to haem iron, such as pyridine and also proteins, especially those such as globin and serum albumin which are known to bind haem strongly. Dimerization may therefore be an important and hitherto unrecognized factor in studies of haem catabolism (i.e. the opening of the porphyrin ring system at one of the methene bridges to yield bile pigment). In this context, the work of O'Carra & Colleran (1969) is particularly significant, since it showed clearly that with the ascorbate–oxygen system the rate of haem degradation was much greater for mixtures of myoglobin and protoferrihaem than the sum of the rates with each haem source treated separately (Fig. 1). This rate enhancement was explained in terms of apoprotein catalysis. We have confirmed these experimental findings and have also measured the rate of haem degradation (in terms of bile pigment formed) by using deuteroferrihaem alone, deuteroferrihaem+myoglobin and protoferrihaem+bovine serum albumin (Fig. 1).

Inspection of Fig. 1(*a*) clearly shows that, at equal concentrations, the rate of deuteroferrihaem degradation is much greater than that of protoferrihaem. It is possible that this is directly attributable to the higher monomeric content of the deuteroferrihaem solution and that monomeric ferrihaems are degraded by this system much faster than are dimeric ferrihaems. It is also possible therefore that the enhancement of reactivity of protoferrihaem degradation on addition of myoglobin observed by O'Carra & Colleran (1969) is due, not to apoprotein catalysis, but to an increase in the proportion of monomeric ferrihaem present, induced by myoglobin. The precise way in which this occurs is not clear at present, though it could be by reaction of

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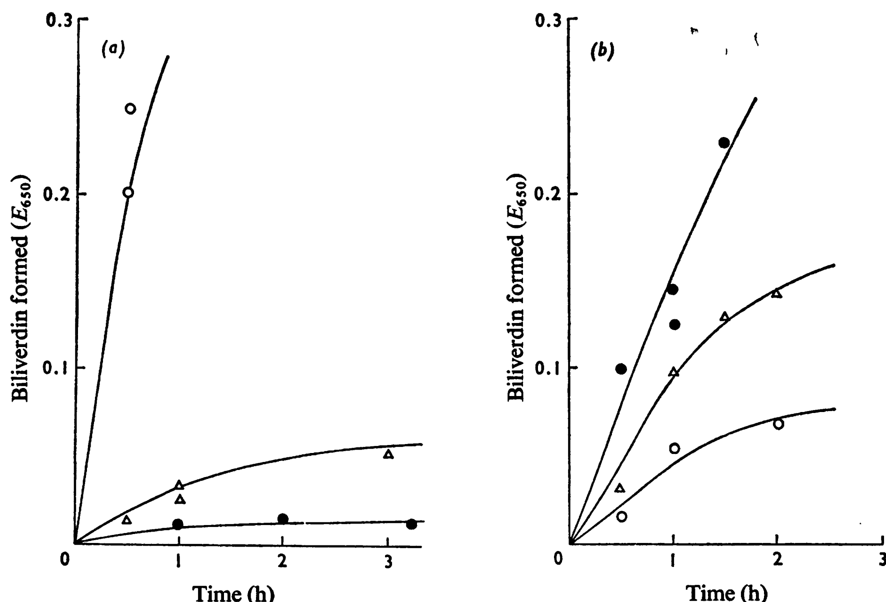

Fig. 1. *Degradation of haem solutions containing various proportions of monomers*

Measurements were made by the method of O'Carra & Colleran (1969). Samples (see below) of the haem source with or without added protein were incubated aerobically with 10mg of ascorbate in 50ml conical flasks at 37°C and pH7 (0.1M-phosphate buffer). The volume of the final reaction mixture was always 7ml. After appropriate times, the incubation mixtures were cooled in an ice bath, 3ml of acetic acid and 8ml of 5M-HCl were added, and the remaining ferrihaem was extracted quickly into peroxide-free ether (2×15ml). The biliverdin was extracted into chloroform (5ml) (reaction mixtures containing protein required centrifugation at this point) and evaporated to dryness under a stream of N_2. The solid was redissolved in 2ml of chloroform and the absorption at 650nm read in a 1cm-light-path cuvette. (*a*) ●, 0.92μmol of protoferrihaem; △, 0.92μmol of deuteroferrihaem; ○, 0.92μmol of protoferrihaem+0.15μmol of bovine serum albumin. (*b*) ●, 0.92μmol of deuteroferrihaem+0.15μmol of myoglobin; △, 0.92μmol of protoferrihaem+ 0.15 μmol of myoglobin; ○ 0.15μmol of myoglobin.

monomeric ferrihaem and apoprotein to re-form myoglobin, the endogenous haem first having been degraded. This is essentially the mechanism suggested by O'Carra & Colleran (1969). However, so far as the rate enhancement is concerned, the myoglobin may be regarded simply as a 'dimer breaker' and not as a specific 'enzyme' for haem breakdown.

This view is supported by experiments carried out with the addition of bovine serum albumin to ferrihaem solutions before degradation. Spectroscopic changes resulting from addition of serum albumin to protoferrihaem are shown in Fig. 2. Comparison with known monomer and dimer spectra (Fig. 2) shows that these changes are in the direction of increased monomer content. Fig. 1(*a*) shows that, on addition of serum albumin, a large enhancement of rate of protoferrihaem degradation is obtained. This rate enhancement may result from the increased proportion of the reactive monomeric species due to binding of ferrihaem to the albumin. Clearly these results would not be expected if the action of myoglobin was a specific enzymic one.

It is significant that experiments using spleen or liver homogenates to investigate haem degradation (haem oxygenase activity) have usually involved addition of serum albumin to the ferrihaem substrates. As above, this result may be interpreted in terms of the necessary monomerization of protoferrihaem, before degradation.

Although protoferrihaem is not degraded significantly in aqueous solutions containing ascorbate, reaction occurs readily in such solutions on addition of pyridine, when the pyridine haemochrome is formed (Lemberg & Legge, 1949). It seems possible that this is primarily due to the action of pyridine as a 'dimer breaker' acting in much the same manner as myoglobin and albumin and is not due to any special property of the pyridine associated with its aromatic delocalized structure. It might be expected that any substance would initiate haem degradation provided

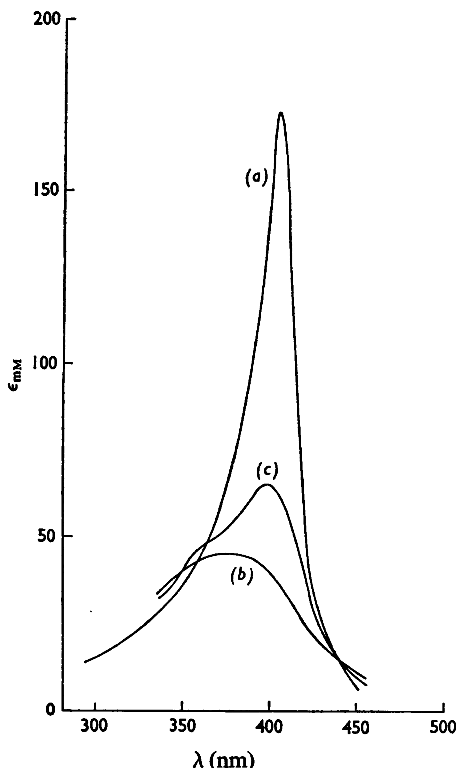

Fig. 2. *Spectra of protoferrihaem*

(*a*) Spectrum of protoferrihaem in dimethyl sulphoxide. This is a typical spectrum of monomeric protoferrihaem. Values of extinction coefficient are independent of ferrihaem concentration. (*b*) Spectrum of protoferrihaem in 0.1 M-phosphate buffer at pH7 (25°C). The protoferrihaem concentration was 50 μM. Under these conditions, protoferrihaem solutions contain 98.5% dimers. This is therefore effectively the spectrum of the protoferrihaem dimer. (*c*) Spectrum of protoferrihaem (50 μM) in 0.1 M-phosphate buffer at pH7 (25°C), with addition of bovine serum albumin (100 μM). This spectrum was obtained 8h after addition of albumin, after which reaction appeared to be complete.

that it was a strong enough ligand to dissociate the ferrihaem dimer and did not otherwise interfere with the reaction. This view is further supported by experiments that we have carried out in dimethyl sulphoxide, a solvent which has been shown to be a good ligand for binding at haem iron (Brown & Lantzke, 1970), but which is not delocalized and is quite unlike pyridine in structure. The spectrum of protoferrihaem in dimethyl sulphoxide (Fig. 2) is typical of monomeric ferrihaems. By using the ascorbate–oxygen system, the rate of haem degradation in dimethyl sulphoxide was found to be comparable with that in pyridine.

Two possible explanations are suggested as to why dimeric ferrihaem should be much less reactive than the monomeric species. First, if, as has been suggested (Kench, 1954), iron performs an essential function in the degradation reaction, it would be reasonable to suppose that formation of an Fe–O–Fe bond in the dimer would perturb the iron environment sufficiently to interfere with this function, thereby diminishing or abolishing reactivity. A second explanation is that the dimer may be unreactive because of the mutual steric influence of the ferrihaem units, one upon the other. Thus the methene bridges of one ferrihaem unit of the dimer may be more or less exactly overlapped by those of the other, thereby providing maximum steric interaction.

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