The Mechanism of Haem Catabolism

A STUDY OF HAEM BREAKDOWN IN SPLEEN MICROSOMAL FRACTION AND IN A MODEL SYSTEM BY ^{18}O LABELLING AND METAL SUBSTITUTION

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The mechanism of bile-pigment formation from haem breakdown was studied by using ^{18}O labelling of the molecular oxygen required for macrocyclic ring cleavage. For haem degradation by the spleen microsomal haem oxygenase system, mass spectrometry of the product bilirubin revealed that cleavage occurred by the Two-Molecule Mechanism, i.e. the terminal lactam oxygen atoms in bilirubin were derived from two different oxygen molecules. Similarly, degradation of myoglobin by coupled oxidation with ascorbate and oxygen proceeded via the Two-Molecule Mechanism. Cobalt and manganese complexes of protoporphyrin IX were not degraded by either the haem oxygenase system or the coupled oxidation system. This result suggests that the iron atom possesses unique properties in facilitating porphyrin breakdown.

The reactions shown in Scheme 1 represent the major pathway for haem disposal in mammals, whereby haem (Scheme 1, *a*) is degraded to the bile pigment biliverdin (Scheme 1, *b*) with concurrent elimination of iron and the appropriate methene-bridge carbon atom as CO. Although in many species biliverdin is the final product, it does not accumulate in mammals, since it is readily converted into bilirubin (Scheme 1, *c*) by the NADPH-dependent enzyme biliverdin reductase (EC 1.3.1.24). Because of the asymmetry in the arrangement of the side chains around the porphyrin periphery (Scheme 1, *a*), the methene-bridge carbon atoms designated α , β , γ and δ are not equivalent, and cleavage at these positions can give rise to four bile-pigment isomers. The relevant literature up to 1975 has been extensively reviewed (Lathe, 1972; Jackson, 1974; O'Carra, 1975; Schmid & McDonagh, 1975).

A feature of studies on haem breakdown has been the several different types of degradation system that can be used. It is relatively easy to study gross aspects of haem catabolism in the intact animal and it is significant that biliary bilirubin contains almost exclusively the IX α isomer. It is also possible to study the reaction *in vitro*. In a series of papers (for review see Schmid & McDonagh, 1975), Schmid and co-workers have described a microsomal enzyme named haem oxygenase (EC 1.14.99.3), capable of catalysing haem cleavage. Since crude preparations of the enzyme contain biliverdin reductase, bilirubin is the final product obtained from haem cleavage by haem oxygenase.

In a study using ^{18}O -labelled oxygen alternately in

molecular oxygen and water, Tenhunen *et al.* (1972) showed that both lactam oxygen atoms in bilirubin obtained by the action of microsomal haem oxygenase were derived from molecular oxygen. Insertion of these oxygen atoms by hydrolytic steps in the formation of bilirubin could therefore be excluded for this system.

Haem degradation may also be studied in rather less biological systems. For example, treatment of myoglobin, haemoglobin (Lemberg, 1956; O'Carra & Colleran, 1969; Bonnett & McDonagh, 1973) or methaemalbumin (Lemberg, 1956; Brown *et al.*, 1974) with ascorbate and molecular oxygen readily results in macrocyclic ring cleavage. It appears to be uncertain whether the direct product of this reaction is biliverdin or a green intermediate retaining the iron atom. In any case, acid hydrolysis readily yields biliverdin, which is the final product of this reaction. Treatment of haem compounds with ascorbate and oxygen has become known as coupled oxidation, since both the haem and ascorbate are ultimately oxidized. O'Carra & Colleran (1969) showed that myoglobin yielded exclusively biliverdin IX α on coupled oxidation, whereas haemoglobin yielded about 65% of the α -isomer and 35% of the β -isomer.

It is significant that none of the haem-degrading systems described above appears to have the capacity to cleave metal-free porphyrins (Tenhunen *et al.*, 1968; Brown & Grundy, 1977). However, it is conceivable that porphyrin complexes with metals other than iron that may bind oxygen, for example cobalt, nickel or manganese, might undergo degradation.

Many studies have been made of haem breakdown *in vitro*, by using the systems described above, with the aim of extrapolating any mechanistic conclusions

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Scheme 1. Structure of tetrapyrrolic compounds involved in haem catabolism
M, $-\text{CH}_3$; V, $-\text{CH}=\text{CH}_2$; P, $-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$.

Table 1. ^{18}O incorporation into bilirubin produced by microsomal haem oxygenase

Haem degradation was carried out under an atmosphere containing oxygen of which 31% was $^{18,18}\text{O}_2$ and 69% was $^{16,16}\text{O}_2$. None of the mixed species $^{18,16}\text{O}_2$ was present. Natural bilirubin exhibits a molecular ion at m/e 584. After allowance for naturally abundant isotopes, residual peaks at m/e 586 and 588 correspond to incorporation of one and two ^{18}O atoms respectively. The four mechanisms predict different labelling characteristics in the product bilirubin.

	Mol% of bilirubin		
	m/e 584	m/e 586	m/e 588
Observed	57.8	33.8	8.4
Double Hydrolytic Mechanism	100	0	0
Hydrolytic Mechanism	69	31	0
One-Molecule Mechanism	69	0	31
Two-Molecule Mechanism	47.6	42.8	9.6

to haem catabolism *in vivo*. It is therefore important to have a means of probing the mechanisms in the various systems to determine whether such extrapolation is valid. We have described such a mechanistic probe using ^{18}O -labelled molecular oxygen (Brown & King, 1978). Full details of the method have been given in the previous paper and only those points essential to an understanding of the present work are given here. Assuming that the two terminal lactam oxygen atoms in bilirubin are derived from either molecular oxygen or from solvent water, four mechanisms may be distinguished. In the Double-Hydrolytic Mechanism both lactam oxygen atoms are derived from solvent water. In the Hydrolytic Mechanism, one atom is derived from solvent water and the other from molecular oxygen. The One-Molecule Mechanism involves incorporation of both oxygen atoms from a single oxygen molecule, whereas in the Two-Molecule Mechanism each oxygen atom is derived from a different oxygen molecule. These

mechanisms may be distinguished by carrying out haem degradation under labelled molecular oxygen containing $^{18,18}\text{O}_2$ and $^{16,16}\text{O}_2$ but none of the mixed species $^{18,16}\text{O}_2$. The mass spectrum of bilirubin shows a strong molecular ion (M) at m/e 584 (Fig. 3). After allowance for the naturally abundant isotopes (Brown & King, 1978) residual peaks at m/e 586 and m/e 588 correspond to incorporation of one and two heavy oxygen atoms respectively. Taking 31% enrichment of oxygen as an example, the predicted incorporations at m/e 584, 586 and 588 are shown in Table 1, which shows that all four mechanisms lead to different labelling characteristics. For living rats, Brown & King (1978) showed that bilirubin was produced exclusively by the Two-Molecule Mechanism. In the present work the ^{18}O -labelling technique has been applied to the microsomal haem oxygenase system *in vitro* and to coupled oxidation of myoglobin, primarily with the aim of determining if the mechanism of haem breakdown in these systems is the same as that previously observed *in vivo*. In addition, we report results from experiments using cobalt- and manganese-substituted porphyrins as potential substrates in haem-cleaving systems.

Experimental

Materials and measurements

All materials, including buffers, were as previously described (Brown & King, 1978). Protoporphyrin IX was prepared by hydrolysis of its dimethyl ester as described by Falk (1964, p. 126). The cobalt(II) complex was obtained by addition of cobalt(II) acetate in acetic acid to a solution of protoporphyrin IX in chloroform, under reflux (Falk, 1964, p. 139). The manganese(III) complex was similarly obtained by addition of a solution of manganese(II) acetate in aqueous acetic acid to a hot solution of protoporphyrin IX in acetic acid, followed by autoxidation by shaking with air (Falk, 1964, p. 139). pH measure-

ments were made on a Pye-Unicam 290 pH-meter and spectra were recorded with a Pye-Unicam SP.1800 dual-beam recording spectrophotometer. Mass spectra of bilirubin samples were obtained on an AEI MS9 mass spectrometer by courtesy of Mr. P. Kelly, School of Chemistry, University of Newcastle upon Tyne, as previously described. Mass spectra of gaseous samples were obtained on an AEI MS10 mass spectrometer.

Preparation of spleen microsomal haem oxygenase

Male Wistar rats weighing 300–400 g were killed by concussion and cervical dislocation. Spleens were plunged into ice-cold 0.25 M-NaCl immediately on removal and cut into small pieces. After several washes with 0.25 M-sucrose solution and removal of fat globules, the tissue was homogenized in a Potter-Elvehjem apparatus. Centrifugation at 8500 g for 10 min produced a microsomal-rich supernatant. This was used as a crude haem oxygenase solution. Haem oxygenase activity was assayed by using two spectrophotometer cuvettes (path length 10 mm, volume 3 ml), maintained at 37°C. To each cuvette 0.5 ml of spleen homogenate supernatant (containing 5–10 mg of protein/ml), 0.1 ml of methaemalbumin solution (151 μ M-human serum albumin+2 mM-haem) and 0.1 M-phosphate ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) buffer, pH 7.45 (2.1 ml in sample cuvette, 2.3 ml in reference cuvette), was added. After an initial 10 min stabilization period to check any fluctuation in absorbance, reaction was initiated by addition of 0.2 ml of 2.5 mM-NADPH to the sample cuvette. Increase in A_{468} corresponding to bilirubin formation was recorded automatically on the spectrophotometer chart recorder. Over the first 5 min of reaction, the increase in A_{468} was linear with time, as shown in Fig. 2. Haem oxygenase activity was measured from the slopes of such graphs (for bilirubin, ϵ_{mM} at 468 nm = 30; Tenhunen *et al.*, 1968). Protein content of spleen microsomal preparations was determined by the biuret reaction (Gornall *et al.*, 1949) with bovine serum albumin as standard.

^{18}O -labelling experiments

The specially constructed apparatus shown in Fig. 1 was used to carry out haem degradation in closed systems containing ^{18}O . The detailed use of this equipment is described in the legend to Fig. 1. For degradation by spleen microsomal fraction, a homogenate from six rats (approx. 22 ml) and NADPH (5 mg) were placed in the main section of the reaction vessel and 1 ml of methaemalbumin solution was placed in the side arm. After degassing and introduction of ^{18}O , the system was allowed to attain thermal equilibrium at 37°C and reaction was initiated by tipping the contents of the side arm into

the base of the flask. Gas samples were taken at the beginning and end of the reaction. The reaction was terminated by removal of the reaction mixture and

Fig. 1. Apparatus used for haem breakdown in an ^{18}O -enriched atmosphere

Pure $^{18,18}\text{O}_2$ was supplied in a 100 ml flask (A) containing a capillary seal (B). Before starting an experiment, a steel bar (C) was placed in the outlet tube and a vacuum tap (D), and ground-glass joint (E), were attached as shown. With the aid of a magnet, the bar C was driven into the capillary, which readily shattered, permitting labelled oxygen to proceed as far as the closed tap (D). The main apparatus consisted of a 1-litre round-bottomed flask fitted with an integral side-arm extension (F) of approx. 20 ml capacity. The design was such as to permit a solution contained in the side arm to be tipped into the main body of the flask. Taps G and H were of the high-vacuum type and controlled the inlets J and K, which were fitted with ground-glass joints. To carry out an experiment under an ^{18}O -enriched atmosphere, the components of the reaction mixture were placed separately in either the side arm or the main flask (see the text), which contained a magnetic stirring bar. The flask containing ^{18}O was attached to the main apparatus via the joints E and K and the solutions were degassed by applying a vacuum pump to the outlet J for approx. 10 min with tap H open. This tap was then closed and tap G opened to admit labelled oxygen to the apparatus, after which tap G was closed and flask A removed. For higher enrichments of ^{18}O a second 100 ml quantity of labelled oxygen could be added in the same way. A bubble trap (L) containing water was attached to joint J and tap H briefly opened so that a controlled stream of air could be admitted to the apparatus to bring the total pressure to atmospheric. Enrichment values could then be calculated from the known volume of the apparatus. Tap H was closed and the bubble trap removed. Reaction was initiated by tipping the contents of the side arm into the main flask, and the mixture was stirred magnetically throughout the incubation period. Gas samples could be taken at any time by attachment of evacuated sample vessels to joint J.

extraction with chloroform (2×15 ml). The emulsion thus obtained was resolved by centrifugation in a bench centrifuge at full speed for 10 min, yielding a solution of bilirubin in chloroform. This was washed twice with water, dried by passage through filter paper (Whatman no. 40) and crystallized as previously described (Brown & King, 1978). A similar procedure was carried out without ^{18}O labelling, to provide a reference unlabelled bilirubin sample for mass spectrometry.

Coupled oxidation under oxygen containing ^{18}O was carried out in the same apparatus. After degassing and admission of labelled oxygen, a solution of sodium ascorbate (320 mg dissolved in 3 ml of 0.1 M-phosphate buffer, pH 7.05), contained in the side arm, was tipped into myoglobin solution (72 mg dissolved in 177 ml of 0.1 M-phosphate buffer, pH 7.05) contained in the main section of the flask. Incubation was allowed to proceed with shaking at 37°C for 5 h. Gas samples were again taken at the beginning and end of the experiment. The flask was cooled in ice and 85 ml of acetic acid added, followed immediately by 180 ml of 5 M-HCl. Unchanged haem was removed by extraction with peroxide-free ether (2×50 ml) and the residual pigment extracted into chloroform (1×100 ml, 1×30 ml). These extracts were pooled, dried by passage through filter paper (Whatman no. 1) and evaporated to dryness under a stream of N_2 . The bile pigment thus obtained was stored overnight in a desiccator before use. The ^{18}O -labelled pigment was dissolved in 0.5 ml of 0.1 M-phosphate buffer (pH 7.05) and added to 2.5 ml of 0.15 M-NaCl before intravenous injection into a male Wistar rat with a bile-duct cannula. Bile was collected continuously over a period of 2 h as described previously (Brown & King, 1978), from which bilirubin was crystallized.

Results and Discussion

Haem degradation by spleen microsomal fraction

Fig. 2 shows results from an experiment to determine the haem oxygenase activity of a typical spleen microsomal preparation (the data shown in Fig. 2 actually refer to the microsomal preparation used in the ^{18}O -labelling experiment). The activity calculated from these data in terms of bilirubin production is 0.077 nmol/min per mg of spleen protein. Extraction and crystallization of bilirubin produced by the action of spleen microsomal fraction on methaemalbumin and described above yielded some $100\text{ }\mu\text{g}$ of product. A similar yield was obtained when the incubation was carried out in the closed system in preparation for an experiment using labelled oxygen. The mass spectrum of the bilirubin obtained in this way is shown in Fig. 3.

Table 1 shows the labelling characteristics of bili-

rubin produced from reaction between microsomal haem oxygenase and methaemalbumin under a constant atmosphere containing oxygen of which 31% was labelled with ^{18}O . The data demonstrate clearly that for the bilirubin produced by this system extensive incorporation of label occurs at both m/e 586 and 588. These results are therefore in agreement with those of Tenhunen *et al.* (1972), which demonstrated that both terminal oxygen atoms in bilirubin were derived from molecular oxygen. However, the present results permit a distinction between the One-Molecule Mechanism and the Two-Molecule Mechanism, not possible in this earlier work. It is clear that bilirubin is produced by spleen microsomal fraction *in vitro* by a Two-Molecule Mechanism. However, the quantitative agreement between the observed proportions of bilirubin at m/e 584, 586 and 588 and those predicted for 31% enrichment with ^{18}O is rather less satisfactory than for living rats (Brown & King, 1978). It is noteworthy that the results in Table 1 refer to a total recovery of ^{18}O of about 25%. The discrepancy between this value and the 31% enrichment in the oxygen atmosphere is too great to be due to a kinetic isotope effect. It appears that there has been some dilution of the ^{18}O -labelled sample by unlabelled bilirubin. Since the total yield of crystalline bilirubin was very small (about $100\text{ }\mu\text{g}$), it is likely that this dilution may be accounted for by unlabelled

Fig. 2. Haem oxygenase assay

The enzyme was assayed by measuring the change in A_{468} of a reaction mixture containing spleen microsomal fraction, methaemalbumin and NADPH in phosphate buffer relative to a similar mixture without NADPH (for details, see the text). After a period of 7 min to allow stabilization of the trace, reaction was initiated by addition of NADPH to the reaction cuvette (arrow and broken line on trace). The increase in A_{468} corresponding to bilirubin formation was linear with time over at least the next 5 min. Enzyme activity was calculated from the slope of this trace. The data shown in the Figure refer to the spleen microsomal preparation used in ^{18}O experiments.

Fig. 3. Mass spectra of natural and ^{18}O -labelled bilirubin. Mass spectra of bilirubin obtained by the action of spleen microsomal haem oxygenase utilizing (a) unlabelled oxygen or (b) oxygen containing 31% $^{18}\text{O}_2$ are shown. With sample (b), the mass spectrum clearly shows incorporation of label at both m/e 584 and 586.

bilirubin that was present in the system or was in the process of being formed before exposure to labelled oxygen. If this is the case the total amount of such unlabelled bilirubin would need to be only about $15\text{ }\mu\text{g}$. In any event, there is little doubt that the Two-Molecule Mechanism operates in this system.

Gas samples taken at the beginning and end of the reaction revealed the presence only of $^{16,16}\text{O}_2$ and $^{18,18}\text{O}_2$, and the results clearly show that no mixed molecules, $^{18,16}\text{O}_2$, are formed in this system. The observation of a Two-Molecule Mechanism cannot therefore be an artifact caused by 'scrambling' of the labelled and unlabelled oxygen molecules. These gas samples were also used in an attempt to monitor production of CO during the reaction. Production of C^{16}O and C^{18}O should lead to peaks in the mass spectrum at m/e 28 and 30 respectively. Measurements at m/e 28 are complicated by the presence of N_2 , but such complications do not apply to m/e 30. These measurements revealed a definite increase in

the peak at m/e 30 at the end of the reaction corresponding to production of C^{18}O , although the precise amount formed could not be measured quantitatively. This observation is in agreement with the data of Tenhunen *et al.* (1972), who also found production of ^{18}O . However, calculations based on the amount of bilirubin formed show that the amount of CO expected to be produced concurrently is so small as to be outside the limits of measurements by the mass-spectrometric techniques available. The observation of an increased peak at m/e 30 could be accounted for by formation of CO in the mass spectrometer by reaction of residual carbon compounds with the oxygen in the sample. Such reactions were found to occur very readily during trial experiments. In principle a correction may be applied by running a blank at the same oxygen concentration as the gas sample. However, in practice, the value of the correction thus obtained is somewhat variable and, in the present work, is large compared with the amount of CO expected in the sample. A further reaction that may produce relatively large amounts of CO in the sample is lipid peroxidation occurring in the spleen microsomal preparation. Such a process has been described by De Matteis *et al.* (1977). In view of these complications, the origin of the oxygen in the CO formed during haem degradation must remain uncertain, although it would be surprising if it were not molecular oxygen.

Cobalt protoporphyrin IX and manganese protoporphyrin IX were tested as possible substrates for spleen microsomal haem oxygenase. Since in these complexes the porphyrin ligand is identical with that in the natural iron complex, any degradation might be expected to produce bilirubin and the reaction could be followed in the usual way by measuring the increase in A_{468} . Experiments were performed both with and without the addition of serum albumin. However, neither the cobalt nor the manganese complex gave rise to any detectable bilirubin with preparations that possessed full activity towards the iron complex. It may therefore be concluded that the cobalt and manganese complexes of protoporphyrin IX are not substrates for haem oxygenase and that iron appears to be essential.

Haem degradation by coupled oxidation

Coupled oxidation of myoglobin with ascorbate and oxygen yields biliverdin IX α as the final product, since this system does not contain the biliverdin reductase necessary for production of bilirubin. Although it was perfectly feasible to carry out coupled oxidation under an ^{18}O -enriched atmosphere, the product biliverdin was not sufficiently volatile for direct mass-spectral analysis. Even when the biliverdin was converted into its dimethyl ester, satisfactory mass spectra suitable for quantitative analysis

Table 2. ^{18}O incorporation into bilirubin produced by rats to which ^{18}O -labelled biliverdin had been administered. Haem degradation was carried out under an atmosphere containing oxygen of which 48% was $^{18,18}\text{O}_2$ and 52% was $^{16,16}\text{O}_2$. None of the mixed species, $^{18,16}\text{O}_2$, was present. The biliverdin obtained was injected intravenously into a male Wistar rat (see the text), from which ^{18}O -labelled bilirubin was obtained.

	Mol% of bilirubin			Mol% at m/e 586
	m/e 584	m/e 586	m/e 588	Mol% at m/e 588
Observed (0–2h bile fraction)	71.7	18.2	10.1	1.80
Double Hydrolytic Mechanism	100	0	0	—
Hydrolytic Mechanism	52	48	0	∞
One-Molecule Mechanism	52	0	48	0
Two-Molecule Mechanism	27.0	50.0	23.0	2.17

could not be obtained. This was probably due to difficulties in crystallization, owing to the small quantities of biliverdin involved. As an alternative procedure, biliverdin obtained from coupled oxidation of myoglobin was administered intravenously to a rat and the resultant bilirubin isolated from the rat bile. Barrowman *et al.* (1976) have shown that, after intravenous administration of biliverdin IX α , the great majority of the resultant bilirubin excreted in the bile appears in the first 2h. This result has been confirmed in this laboratory (C. D. Wood, unpublished work). Bilirubin was therefore crystallized from bile obtained from 0 to 2h after injection of biliverdin. However, since concurrent production of endogenous bilirubin may be expected to lead to considerable isotope dilution of bilirubin produced from administered biliverdin, a relatively high ^{18}O enrichment (48%) was used in an ^{18}O -labelling experiment, the results from which are shown in Table 2. It is evident that incorporation has occurred at both m/e 586 and 588, suggesting the Two-Molecule Mechanism.

Although Table 2 lists the quantitative predictions for the various mechanisms, clearly these values could never be attained in practice, because dilution by endogenous bilirubin leads to an increase in the mol% of bilirubin at m/e 584 and decreases in the values at m/e 586 and 588, compared with the predicted values. However, the ratio of the predicted mol% values at m/e 586 and 588 ought to be unaffected by this dilution. The observed ratios along with the predicted values are shown in Table 2. It is clear that the ratio is close to that predicted for the Two-Molecule Mechanism. The total ^{18}O recovered in this fraction was 19.2%, which corresponds to a dilution of the administered bile pigment by endogenous bile pigment of approx. 1.5:1.

The cobalt and manganese complexes of protoporphyrin IX were also tested as possible substrates for coupled oxidation with ascorbate in aqueous pyridine. Under these conditions, ring cleavage of the iron complex occurs rapidly. Possible porphyrin

breakdown was investigated by monitoring absorption of the metal porphyrin at wavelengths of the visible absorption maxima (540 and 574nm for the cobalt complex and 475 and 558nm for the manganese complex). However, no changes in absorption occurred at these wavelengths. In addition, the A_{650} corresponding to the absorption maximum of the haem-cleavage product showed no increase. Prior addition of sodium dithionite to the reaction mixture had no effect. From these experiments it must be concluded that the cobalt and manganese complexes of protoporphyrin IX are not susceptible to coupled oxidation and that, as with the spleen microsomal system, iron is required.

General discussion

Two-Molecule Mechanisms have been observed in the systems described in the present work and also in the intact animal (Brown & King, 1978). This finding gives confidence in the reliability of the systems *in vitro* as models for haem catabolism in the intact animal, a conclusion that is reinforced by the recent observation (S. B. Brown & B. D. Chaney, unpublished work) that coupled oxidation of octaethylhaem in pyridine also proceeds via a Two-Molecule Mechanism. It appears likely that haem breakdown in non-photochemical systems proceeds via a unique mechanism that is independent of the solvent system or the influence of proteins, insofar as the nature of the oxidation steps is concerned. However, it is clear that a protein component is essential in directing the methene-bridge selectivity of the reaction (Brown, 1976).

Since the Two-Molecule Mechanism has been observed in all haem degradation systems so far studied, it has proved possible to eliminate certain otherwise plausible intermediates from the degradation pathway (Brown & King, 1975, 1978). However, it has not yet been possible to suggest a complete and adequate molecular pathway consistent with a Two-Molecule Mechanism and the elimination of the

methene-bridge carbon atom as CO. This problem has been discussed in detail by Brown & King (1978).

From the present work and previous studies (Brown & Grundy, 1977; Jackson, 1974) it seems clear that iron is essential for haem cleavage. This reinforces the concept (Brown, 1976) that degradation occurs via an attack by an iron-bound activated oxygen molecule at a methene-bridge carbon atom. Although the precise nature of the activated oxygen is not known, it seems likely that this is at the peroxide level produced by electron transfer mediated by the iron atom and an appropriate reducing agent. It is significant that a reducing agent is always required for haem oxidation by molecular oxygen. This role is performed by ascorbate in the more chemical system and by NADPH and the microsomal electron-transport system *in vivo*.

It is somewhat surprising that the cobalt-porphyrin complex was not degraded, since such complexes are known to bind molecular oxygen as, for example, in the cobalt haemoglobin analogues. However, it may be that such compounds, although binding oxygen, do not possess the oxidase characteristics of the iron haemoproteins. It is possible that, superimposed on its ability to bind molecular oxygen, iron may be unique in possessing exactly the required electron-transfer properties to produce intramolecular activated oxygen.

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References

- Barrowman, J. A., Bonnett, R. & Bray, P. J. (1976) *Biochim. Biophys. Acta.* **444**, 333–337
- Bonnett, R. & McDonagh, A. F. (1973) *J. Chem. Soc. Perkin Trans.* 881–888
- Brown, N. A., King, R. F. G. J., Shillcock, M. S. & Brown, S. B. (1974) *Biochem. J.* **137**, 135–137
- Brown, S. B. (1976) *Biochem. J.* **159**, 23–27
- Brown, S. B. & Grundy, M. S. (1977) *Biochem. Soc. Trans.* **5**, 1017–1020
- Brown, S. B. & King, R. F. G. J. (1975) *Biochem. J.* **150**, 565–567
- Brown, S. B. & King, R. F. G. J. (1978) *Biochem. J.* **170**, 299–311
- De Matteis, F., Gibbs, A. H. & Unseld, A. (1977) *Biochem. J.* **168**, 417–422
- Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam
- Gornall, A. G., Bardawill, J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Jackson, A. H. (1974) in *Iron in Biochemistry and Medicine* (Jacobs, A. & Worwood, M., eds.), pp. 145–182, Academic Press, London and New York
- Lathe, G. H. (1972) *Essays Biochem.* **8**, 107–121
- Lemberg, R. (1956) *Rev. Pure Appl. Chem.* **6**, 1–23
- O'Carra, P. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., ed.), pp. 123–153, Elsevier, Amsterdam
- O'Carra, P. & Colleran, E. (1969) *FEBS Lett.* **5**, 295–298
- Schmid, R. & McDonagh, A. F. (1975) *Ann. N.Y. Acad. Sci.* **244**, 533–552
- Tenhunen, R., Marver, H. S. & Schmid, R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 748–755
- Tenhunen, R., Marver, H. S., Pimstone, N. R., Trager, W. F., Cooper, D. Y. & Schmid, R. (1972) *Biochemistry* **11**, 1716–1720