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THE MECHANISM OF HAEM DEGRADATION

AN ^{18}O STUDY OF HAEM COUPLED OXIDATION AT LOW OXYGEN PARTIAL PRESSURE

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Abstract Coupled oxidation of protohaem with ascorbate and molecular oxygen was studied under conditions of low oxygen partial pressure. Reaction conditions were established whereby a reasonable extent of reaction was achieved without total depletion of the oxygen present. Under these conditions, experiments with $^{18,18}O_2/^{16,16}O_2$ mixtures failed to reveal the presence of the isotopically scrambled species $^{18,16}O_2$ following coupled oxidation. Under the same conditions, no H_2 $^{18,16}O_2$ was detected in solution following reaction. These data suggest that a mechanism for haem degradation involving evolution of a dioxygen species cannot account for the experimental observation of a Two-Molecule Mechanism whereby the lactam O atoms in the product bile pigment are derived from each of two oxygen molecules. An alternative mechanism is proposed.

THE oxidative cleavage of the haem macrocycle to yield a bilin plays a significant part in the metabolism of both animals and plants. In mammals and other animals containing haemoglobin, the degradation of haem to biliverdin (Fig. 1) and, in some cases to bilirubin (**L6**), is important because haemoglobin is relatively abundant and the metabolic flux along the haem-bile pigment pathway is correspondingly high. Considerable interest has been shown in the mechanism of haem degradation partly because of the clinical problems which can arise due to excess bilirubin production or its inefficient removal. The area has been extensively reviewed both from clinical and chemical viewpoints.[1-6]

Mammalian bile pigments appear to serve no function other than providing a pathway for the elimination of unwanted haem. By contrast, plants and blue-green algae contain bilins which perform important functions in photosynthesis and photomorphogenesis. These plant bilins are very similar in structure to the mammalian bile pigments, as exemplified by the structure of phycocyanobilin (Fig. 1) the chromophore of the photosynthetic antennae pigment in certain algae. Recently, it has been shown using ^{14}C labelling that, like the mammalian bile pigments, phycocyanobilin is formed via haem.[7]

Haem degradation to bilins may also be studied in non-biological systems. For example, treatment of haem in aqueous pyridine with ascorbate in the presence of O_2 results in formation of biliverdin,[8] which is probably initially formed as its iron complex. This so-called coupled oxidation may also be applied to the degradation of the haem in haemoproteins. In the absence of protein, an almost random mixture of all four biliverdin isomers is produced,[9,10] but coupled oxidation of haemoproteins results in considerable methine bridge selectivity.[5]

The first intermediate formed in the haem cleavage

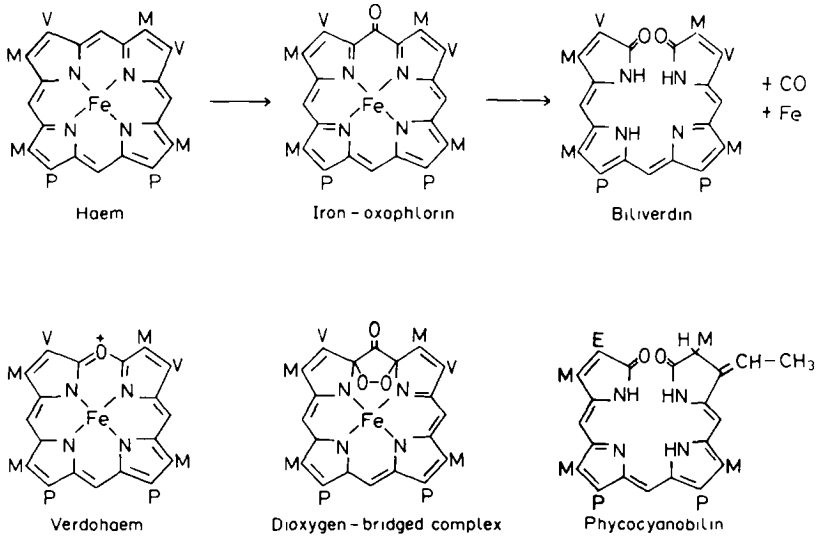

Fig. 1. Haem degradation to bile pigment and structure of some related compounds (M, $-CH_3$; V, $-CH{=}CH_2$; P, $CH_2CH_2CO_2H$; E, $-CH_2CH_3$).

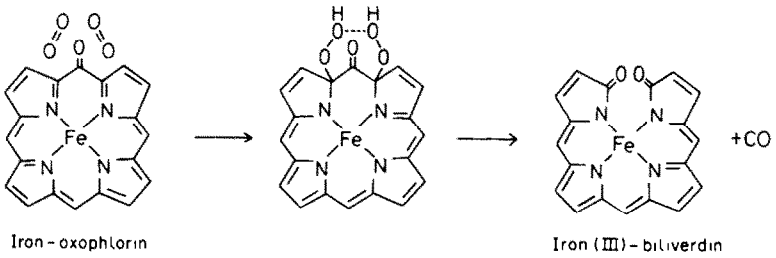

Fig. 2. Haem degradation – possible mechanism involving dioxygen evolution (side chains are omitted).

process is probably the iron oxophlorin complex shown in Fig. 1. Although this compound has never been isolated from biological systems, there is strong circumstantial evidence for its formation (for review see ref. 6). Among this evidence is the finding that intravenously administered iron oxophlorin is rapidly metabolised to the corresponding biliverdin.[11] In addition, iron oxophlorins are formed in chemical systems in which haem is degraded to bile pigment.[12,13] Treatment of these complexes with molecular oxygen alone results in formation of the corresponding bilin, although the yield is considerably increased by treatment with oxygen in the presence of ascorbate.[6]

The mechanism of the macrocyclic ring cleavage step has been studied in detail by ^{18}O labelling. For the enzyme-catalysed degradation of haem, Tenhunen *et al.*[14] showed that both lactam oxygen atoms incorporated into bilirubin were derived from molecular oxygen. These initial experiments have been extended by carrying out haem degradation under atmospheres containing mixtures of $^{18,18}O_2$ and $^{16,16}O_2$ but none of the mixed species $^{18,16}O_2$. Following isolation and mass spectrometry of the product bilin, it was thereby possible to determine whether the lactam O atoms were derived from a single oxygen molecule (One-Molecule Mechanism) or from two oxygen molecules (Two-Molecule Mechanism). Such experiments have now been carried out on living rats producing bilirubin,[15,16] living plant cells synthesising phycocyanobilin,[17,18] the haem oxygenase enzyme system *in vitro*,[19] the coupled oxidation of haemoproteins,[19] pyridine haemochrome coupled oxidation[20] and the oxidation, with and without ascorbate, of iron octaethyloxophlorin.[21] In every case, a Two-Molecule Mechanism has been found, suggesting that all haem degradation reactions, whether biological or non-biological, proceed by the same mechanism. During all of these studies, the total oxygen partial pressure was between 0.2 and 1.0 atmosphere and the dimensions of reaction vessels were such that oxygen was in significant molar excess over the bilin produced. Gas phase analyses carried out after the reactions revealed no significant accumulation of the mixed $^{18,16}O_2$ species, compared with $^{18,18}O_2$ and $^{16,16}O_2$.

The observation of a Two-Molecule Mechanism has important implications for the mechanism of the ring cleavage step. Some previously postulated intermediates such as verdohaemochrome[8] (Fig. 1) and the dioxygen-bridged intermediate[2] (Fig. 1) are no longer considered likely. Verdohaemochrome is probably formed as a side reaction after production of the iron-biliverdin complex by ring closure,[6] although it has recently been suggested[22] that it could be reconverted to biliverdin via an oxidation-reduction reaction as well as by a hydrolytic mechanism. The dioxygen-bridged complex would presumably require a One-Molecule Mechanism for conversion to biliverdin. Although the observation of a Two-Molecule Mechanism has rendered these previously postulated intermediates unlikely, a precise molecular explanation by which a Two-Molecule Mechanism can be achieved has proved elusive.

The need to explain the Two-Molecule Mechanism in molecular terms has led us to consider the scheme shown in Fig. 2. In this mechanism, two molecules of oxygen react independently and sequentially with the iron oxophlorin but, by an internal rearrangement as shown, a molecule of dioxygen is then released to yield the iron biliverdin complex and CO (the released dioxygen might alternatively be produced as peroxide). The scheme in Fig. 2 would be consistent with the Two-Molecule Mechanism and can potentially be evaluated experimentally, since the dioxygen released would contain an atom from each of the two original oxygen molecules reacting. For reaction occurring under a mixture of $^{18,18}O_2$ and $^{16,16}O_2$ this implies a scrambling process with production of the mixed species $^{18,16}O_2$. However, this would be produced in stoichiometrically equivalent amounts to the haem or iron oxophlorin degraded and might not be detectable under the conditions of large oxygen excess used previously. To evaluate the possibility of the scheme in Fig. 2, it is therefore necessary to carry out the macrocyclic ring cleavage step under conditions of low oxygen partial pressure in a relatively small reaction vessel and to examine the gas phase after reaction for the presence of $^{18,16}O_2$. The present work describes the development of systems in which haem degradation may be carried out under such conditions.

EXPERIMENTAL

O_2 gas containing 99 atom % ^{18}O was obtained from Prochem, The British Oxygen Co Ltd, Deer Park Road, London, SW19 3UF, U.K. Protohaemin chloride and pyridine were of analytical reagent grade supplied by BDH Chemicals, Poole, Dorset, U.K. Pyridine was redistilled before use. Electronic spectra were recorded on a Pye-Unicam SP8-100 spectrophotometer. Mass spectra of gaseous samples were obtained on an AEI MS10 mass spectrometer.

Coupled oxidation under $^{16,16}O_2$ at low oxygen partial pressure

Aqueous pyridine (1 : 1 v/v, *ca* 5 ml) was added to a 25 ml round-bottomed flask and degassed by freezing and thawing on a vacuum line. The line incorporated a manometer (to check for leaks) and attachments for a vacuum pump, a gas sampling vessel, the reaction vessel and a reservoir containing a predetermined mixture of $^{16,16}O_2$ in N_2. Protohaemin (30 mg) and sodium ascorbate (for quantities see Table 1) were added to the frozen degassed soln. The O_2/N_2 mixture was then introduced into the reaction flask such that the

Table 1. Coupled oxidation of protohaem at low oxygen partial pressure

Experiment number	Weight of ascorbate used (mg)	Temperature (°)	Reaction time (min)	Yield of product (μmol)	Residual oxygen after experiment (μmol)
1	30	40	30	36	4
2	20	53	30	26	16
3	10	57	30	20	160
4	15	53	30	32	50
5	15	60	30	30	44
6	15	65	15	35	20
7	15	57	20	18	140

In each experiment 200 μmol oxygen and 30 mg haem were used initially. The yield of product (presumed to be the iron biliverdin complex) was determined as described in the Experimental section. Approximately 5 ml of aqueous pyridine (1 : 1 v/v) was used as solvent for each experiment.

total pressure was 1 atmosphere and a sample from the gas reservoir was taken for analysis by mass spectrometry.

The flask containing the frozen solvent mixture and reactants was rapidly warmed to the required temp and maintained at this temp (with stirring) for the required time. Reaction was stopped by cooling in ice and a sample of the gas phase was removed for mass spectral analysis. A small aliquot of the soln (0.1 ml) was immediately removed and its absorbance measured at 650 nm after appropriate dilution. From this absorbance and the estimated molar absorption coefficient[23] of $20 \times 10^3\ M^{-1}\ cm^{-1}$, a value for the amount of ring-opened product formed could be calculated. The O_2 contents of gaseous samples were determined from the relative heights of peaks due to N_2 and $^{16,16}O_2$ in their mass spectra. Using this information and a knowledge of the volume of the reaction vessel, the amount of O_2 remaining after each experiment could be estimated as shown in Table 1.

The experiment was repeated varying weight of ascorbate, reaction temp and time. Table 1 lists the reaction conditions for each experiment.

Coupled oxidation under $^{18,18}O_2/^{16,16}O_2$

A similar procedure to the above was adopted for ^{18}O labelling experiments. Before commencement of a run, pure (99%) $^{18,18}O_2$ was mixed with an equal volume of $^{16,16}O_2$ using a method previously described,[16] and this was mixed with N_2 such that the final mixture consisted of 5% (v/v) $^{18,18}O_2$, 5% (v/v) $^{16,16}O_2$ and 90% (v/v) N_2. A sample of this mixture was removed for mass spectral analysis. Reaction was carried out as previously described using 30 mg protohaemin and 15 mg ascorbate. Other conditions are indicated in Table 2. After an appropriate time, the mixture was cooled in ice and a sample of the gas phase was collected. At this stage, a large (100 ml) sampling vessel was used to try to remove as much as possible of the remaining O_2, including that dissolved in solvent. The experiment was repeated several times as indicated in Table 2.

In one experiment, the mixture was examined for the presence of H_2O_2. This was done by completely degassing the spent mixture, followed by addition, under vacuum, of solid $KMnO_4$. A gas was evolved rapidly and a sample was taken for mass spectral analysis.

RESULTS

Coupled oxidation at low oxygen partial pressure

Two potential problems are presented by attempts to carry out haem coupled oxidation at low oxygen partial pressures. First, the rate of haem degradation may be significantly reduced and secondly, the direct oxidation of ascorbate may deplete the already low oxygen concentration. This latter reaction, which may be catalysed by haem itself,[24] is known to occur during conventional coupled oxidation, but it then has little significant effect, either because reactions have been carried out in open systems with an unlimited oxygen supply, or in closed systems with oxygen in excess.

Trial experiments were carried out to determine if coupled oxidation of protohaem in aqueous pyridine could be carried out at an acceptable rate and yield of product, without total depletion of oxygen. Clearly, it was essential that sufficient oxygen should remain after the experiment, to permit sampling and mass spectrometry. With the apparatus used, it was found that a gas mixture of O_2/N_2 (1 : 9 v/v) to a total pressure of 1 atmosphere was most appropriate. This effectively fixed the volume and hence number of moles of oxygen employed initially in each experiment. The amount of protohaemin used in each run was also fixed at 30 mg (46 μmol). The results of a number of experiments in which ascorbate concentration, time of incubation and reaction temperature were varied are shown in Table 1. Experiments 1, 2 and 3 confirm that the amount of oxygen used depends critically upon ascorbate concentration. Thus, when 20 mg or 30 mg of ascorbate were used, very little oxygen was left after the incubation; this was insufficient for sampling and mass spectral analysis. Presumably, this was due to oxidation of ascorbate by molecular oxygen, unrelated to haem degradation. When only 10 mg of ascorbate was used (Experiment 3), little oxygen was utilised but the yield of product was correspondingly less. When 15 mg of ascorbate was employed, the amount of oxygen remaining was sufficient for analysis and the extent of reaction was reasonable as measured by the increase in absorption at 650 nm. An increase in temperature (e.g. Experiment 6) resulted in a more rapid reaction and gave a good yield of product, but again resulted in little oxygen remaining. From these data, the most optimum reaction conditions appeared to require 15 mg of ascorbate, a reaction time between 20–30 min and a reaction temperature of 55°.

Coupled oxidation in presence of $^{18,18}O_2$

Several experiments were carried out under an initial oxygen atmosphere consisting of approximately 50% $^{18,18}O_2$ and 50% $^{16,16}O_2$, with a total oxygen partial pressure of 0.1 atmosphere, the remainder of the gas phase consisting of nitrogen. Table 2 (Experiments 1–3) shows data obtained from the gas phase samples before and after reaction. Before the reaction, there is only a very small peak in the mass spectrum

Table 2. Coupled oxidation of protohaem under $^{18,18}O_2/^{16,16}O_2$

Experiment number	Reaction time (min)	Percent oxygen in gas phase before experiment (m/e)			Percent oxygen in gas phase after experiment (m/e)		
		32	34	36	32	34	36
1	20	50.0	0.4	49.6	52.2	0.8	47.0
2	20	50.0	0.4	49.6	49.8	0.9	49.2
3	20	50.0	0.4	49.6	49.8	0.2	50.0
4	35	50.0	0.4	49.6	43.4	1.2	55.4
4		After addition of $KMnO_4$			42.3	0.8	58.3

Reaction temperature was 54°; volume of reaction solution was 5 ml except for Experiment 4, when it was reduced to 1 ml.

at *m/e* 34, amounting for less than 0.5% of the total oxygen present. This is due to the presence, in the $^{18,18}O_2$ gas supplied, of a small trace of $^{18,16}O_2$. After reaction, no significant increase in the mass spectral peak at *m/e* 34 was observed in any of these experiments. Any oxygen which might be evolved according to the scheme in Fig. 1 would, of course, appear initially in the solution phase. Although constant stirring should have ensured rapid equilibration with the gas phase, and a large gas sample was taken in order to collect at least part of the dissolved oxygen, an attempt was made to reduce the possibility that any isotopically scrambled oxygen might be retained in the liquid phase by carrying out an experiment using only 1 ml of solvent (Table 2, Experiment 4). Again however, the gas phase contained no significant increase in the peak at *m/e* 34.

Since no scrambled oxygen was observed following coupled oxidation, it was possible, if unlikely, that any dioxygen released during haem degradation might appear as hydrogen peroxide. This would remain in solution and not be detected by the above procedures. The spent reaction solution from one experiment (Table 2, Experiment 4) was therefore degassed and analysed for the possible presence of $H_2\ ^{18,16}O_2$. After addition of potassium permanganate, a gas was evolved which was shown in the mass spectrometer to be oxygen, implying that hydrogen peroxide was indeed present in the reaction mixture. Mass spectrometric analysis revealed the composition for this evolved oxygen shown in Table 2, which again failed to reveal the presence of any isotopically scrambled oxygen. Hence, the hydrogen peroxide must have consisted of $H_2\ ^{18,18}O_2$ and $H_2\ ^{16,16}O_2$ but none of the mixed species, $H_2\ ^{18,16}O_2$.

DISCUSSION

Failure to observe isotopically scrambled oxygen during these experiments seems to suggest that the mechanism proposed in Fig. 2 cannot operate in haem degradation. However, it is important to be sure that such oxygen would have been detected if it had been formed. Clearly, the amount of scrambled oxygen expected depends upon the extent of reaction. It is known that coupled oxidation of protohaem in pyridine does not give a quantitative yield of bile pigment. In the present work we have estimated the extent of reaction by measurement of the increase in absorption at 650 nm. It has been suggested that this absorption is due to verdohaemochrome,[23] but more recent work suggests that it is probably due to an iron biliverdin complex.[6] Certainly, there is no doubt that the 650 nm absorption refers to a ring-opened compound following elimination of CO and insertion of two atoms of molecular oxygen.[6] The molar absorption coefficient of this species is not known precisely, but an estimate of $20.3 \times 10^3\ M^{-1}\ cm^{-1}$ has been made[23] and this is clearly of the order of magnitude expected for a biliverdin-like compound at its absorption maximum in this wavelength range (cf. biliverdin IX-α in $CHCl_3$, $\varepsilon_{650} = 14.3\ M^{-1}\ cm^{-1}$, phycocyanobilin in $CHCl_3$, $\varepsilon_{600} = 18.9\ M^{-1}\ cm^{-1}$). Although the estimates of yield of ring-opened product in Table 1 may therefore require minor revision when a precise absorption coefficient is available, there is little doubt that the data are sufficiently accurate for the assessment of the minimum extent of reaction, required for the present purpose.

The precise calculations of the expected proportion of scrambled oxygen in the gas remaining after an experiment is difficult for two reasons. First, oxygen is utilised for direct oxidation of ascorbate in addition to its requirement for haem degradation. The relative rates of each reaction are difficult to assess and may vary with time of incubation and reaction conditions. Secondly, any scrambled oxygen produced would join the total oxygen pool and would be used proportionately for both ascorbate oxidation and further haem degradation. Since however, no isotopically scrambled oxygen was observed, an important criterion for assessment of the present experiments is to determine the minimum scrambling expected according to the scheme in Fig. 2. Clearly, if the minimum scrambling expected would be detectable, then the failure to observe $^{18,16}O_2$ is a significant result. The total production of $^{18,16}O_2$ in any experiment would be equal (in terms of moles) to half the yield of product obtained. This would be so because the participation of oxygen containing 50% $^{18,18}O_2$ and 50% $^{16,16}O_2$ in the mechanism of Fig. 2 would produce 25% $^{18,18}O_2$, 50% $^{18,16}O_2$ and 25% $^{16,16}O_2$ in the evolved oxygen. Any recycling of this evolved oxygen would not change the isotope distribution. For a typical yield of about 30 μmol product, the total production of $^{18,18}O_2$ would therefore correspond to 15 μmol. The minimum possible proportion of $^{18,16}O_2$ in the gas phase therefore, corresponds to about 7.5% of the total oxygen present (i.e. 15 μmol as a percentage of the initial 200 μmol oxygen). In reality, the proportion will be considerably higher than this, since $^{16,16}O_2$ and $^{18,18}O_2$ are used preferentially at the beginning of the reaction and eventually, little oxygen remains in

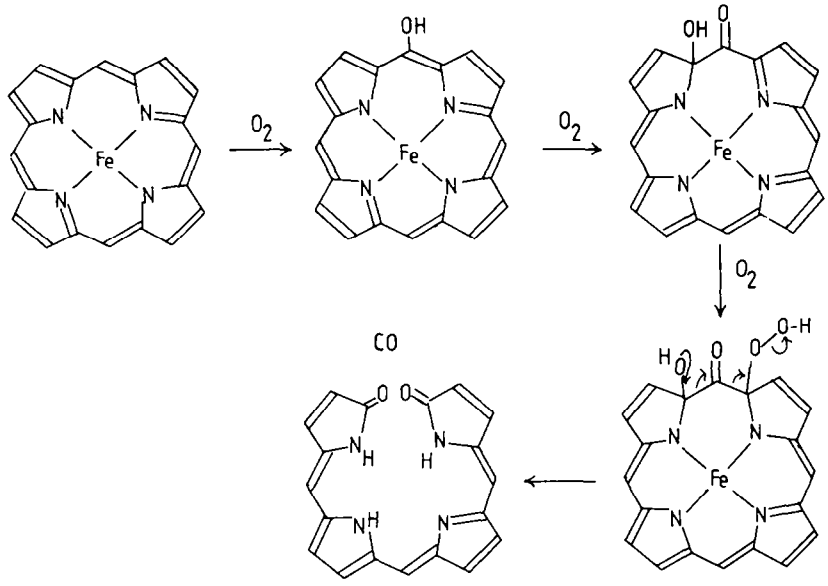

Fig. 3. Possible scheme to account for Two-Molecule Mechanism in haem degradation (side chains are omitted).

some experiments. There is no doubt that peaks in the mass spectrum at *m/e* 34 corresponding to $>7.5\%$ of the total oxygen present would be readily detected.

These considerations suggest that the failure to find isotopically scrambled molecular oxygen is a mechanistically meaningful result and that the scheme suggested in Fig. 2, involving evolution of oxygen, cannot operate in the coupled oxidation. The results also show that, although some hydrogen peroxide was detected, it did not contain $H_2{}^{18,16}O_2$ and therefore could not have arisen from the mechanism in Fig. 2. Presumably, this peroxide was produced from the direct reduction of molecular oxygen by ascorbate. Experiments similar to those reported here have been carried out, in the absence of ascorbate, using iron octaethyloxophlorin as starting material. In these studies also, isotopically scrambled oxygen was not detected.[25,26]

The uniqueness of the haem degradation reaction has been highlighted by studies of seemingly comparable reactions such as the photo-oxidation of tetracyclone[27] and the enzymic and chemical oxidation of quercetin,[28,29] both of which involve ring opening, insertion of two oxygen atoms into the product and elimination of CO. Both of these systems have been shown to proceed via One-Molecule Mechanisms. It may be highly significant that, unlike haem degradation, these systems do not contain a metal atom. This reinforces the postulate that haem degradation proceeds by three essentially independent reactions involving molecular oxygen.[15] The first step utilises one oxygen molecule to yield the iron oxophlorin which is then degraded to bile pigment by two similar reactions to give a Two-Molecule Mechanism. These ideas are supported by the fact that iron is required at all stages of the reaction, i.e. metal-free oxophlorins do not yield bile pigment on treatment with oxygen. The concept of three independent oxidation steps has recently been represented in mechanistic form by Battersby[30] and is shown in Fig. 3. This scheme is attractive since it allows for the requirement of iron in both the initial hydroxylation step and in the ring cleavage steps. In addition, it is consistent with other mechanisms by which CO is produced. At the present time, this seems the simplest and most likely mechanism to account for haem degradation.

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REFERENCES

[1]G. H. Lathe, *Essays Biochem.* **8**, 107 (1972).
[2]R. Schmid and A. F. McDonagh, *Ann. N.Y. Acad. Sci.* **244**, 533 (1975).
[3]R. Schmid and A. F. McDonagh, *The Porphyrins* (Edited by D. Dolphin) Vol. VI, p. 257. Academic Press, New York (1979).
[4]A. H. Jackson, *Iron in Biochemistry and Medicine* (Edited by A. Jacobs and M. Worwood), p. 145. Academic Press, New York (1974).
[5]P. O'Carra, *Porphyrins and Metalloporphyrins* (Edited by K. M. Smith), p. 123. Elsevier, Amsterdam (1975).
[6]S. B. Brown and R. F. Troxler, *Bilirubin Metabolism* (Edited by K. P. M. Heirwegh and S. B. Brown) Vol. 2. CRC Press, Florida (in press).
[7]S. B. Brown, J. A. Holroyd, R. F. Troxler and G. D. Offner, *Biochem. J.* **194**, 137 (1981).
[8]R. Lemberg, *Biochem. J.* **29**, 1322 (1935).
[9]R. Bonnett and A. F. McDonagh, *J. Chem. Soc.*, Perkin Trans. **1**, 881 (1973).
[10]P. O'Carra and E. Colleran, *J. Chromatogr.* **50**, 458 (1970).
[11]T. Kondo, P. C. Nicholson, A. H. Jackson and G. W. Kenner, *Biochem. J.* **121**, 601 (1971).
[12]A. H. Jackson, *Chemistry and Physiology of Bile Pigments* (Edited by P. D. Berk and N. I. Berlin), p. 41. US Department of Health, Education and Welfare, Washington DC (1975).
[13]R. Bonnett and M. J. Dimsdale, *J. Chem. Soc.*, Perkin Trans. **1**, 2540 (1972).
[14]R. Tenhunen, H. S. Marver, N. R. Pimstone, W. F. Trager, D. Y. Cooper and R. Schmid, *Biochemistry* **11**, 1716 (1972).
[15]S. B. Brown and R. F. G. J. King, *Biochem. J.* **150**, 565 (1975).
[16]S. B. Brown and R. F. G. J. King, *Biochem. J.* **170**, 297 (1978).

[17]R. F. Troxler, A. S. Brown and S. B. Brown, *J. Biol. Chem.* **254**, 3411 (1979).
[18]S. B. Brown, J. A. Holroyd and R. F. Troxler, *Biochem. J.* **190**, 445 (1980).
[19]R. F. G. J. King and S. B. Brown, *Biochem. J.* **174**, 103 (1978).
[20]B. D. Chaney and S. B. Brown, *Biochem. Soc. Trans.* **6**, 419 (1978).
[21]A. H. Jackson, M. G. Lee, R. T. Jenkins, S. B. Brown and B. D. Chaney, *Tetrahedron Letters* 5135 (1978).
[22]S. Saito and H. A. Itano, *Proc. Natl. Acad. Sci.* **79**, 1393 (1982).
[23]E. Y. Levin, *Biochemistry* **5**, 2845 (1966).
[24]S. B. Brown, unpublished work.
[25]S. B. Brown and B. D. Chaney, unpublished work.
[26]R. T. Jenkins, Ph.D. Thesis, University of Wales (1980).
[27]B. D. Chaney and S. B. Brown, *Photochem. Photobiol.* **28**, 339 (1978).
[28]S. B. Brown, V. Rajananda, J. A. Holroyd and E. G. V. Evans, *Biochem. J.* **205**, 239 (1982).
[29]V. Rajananda and S. B. Brown, *Tetrahedron Letters* 4331 (1981).
[30]A. R. Battersby, personal communication (1981).