Mechanism of a Novel Synthesis of Haemin *c* from Protohaemin and L-Cysteine. A Markownikoff-type Radical Addition Reaction

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As a simulation of *in vivo* sulphide bond formation of *c*-type cytochromes, haemin *c* (4) was synthesized by the reaction of iron protoporphyrin IX [protohaemin (3)] with sodium borohydride in the presence of L-cysteine, oxygen, and cetyltrimethylammonium bromide (CTAB). When L-cysteine was omitted from the reaction mixture, mesohaemin (9) and haematohaemin (5) were obtained. The inhibitory effect of cyanide anion or carbon monoxide, as well as the inability of protoporphyrin (8) to serve in place of (3) in both reactions, indicated that the iron of (3) and oxygen were crucial in a primary process to give a common intermediate (11) for (4), (5), and (9). Trapping of (11) with oxygen indicated that it is the α-carbon radical of the 2- or 4-ethyl group derived from (3). The addition of deuterium (from sodium borodeuteride) to the β-carbon of the vinyl group of (3) and the resulting formation of (11) strongly suggested the intermediacy of a free hydrogen atom, which was generated in the reduction of (3) with sodium borohydride. The generation of a free hydrogen atom was also supported by a transfer experiment.

c-TYPE cytochromes have sulphide linkages between the α-carbons of the ethyl groups of mesohaemin and two cysteine residues of the apoprotein.[1] Although such a characteristic covalent bond has been known for some time, the mechanism of the *in vivo* sulphide bond formation is still unknown. One model reaction for the formation of the sulphide bond was carried out in neutral aqueous solution at room temperature to try and mimic possible physiological conditions. Thus porphyrin *c* (1) [2,4-di-(α-*S*-cysteinylethyl)deuteroporphyrin IX] was prepared in high yield by the autoxidation of protoporphyrinogen (2), which is a biosynthetic precursor of protohaemin (3), to porphyrin in the presence of L-cysteine.[2,3] This reaction gave a Markownikoff-type adduct despite the fact that a considerable amount of peroxide formation was expected during the autoxidation of (2). This reaction was applied further to prepare a cytochrome *c*-like compound by allowing re-combination of (2) with apocytochrome *c*, followed by iron insertion.[4] Catalytic activity of the reconstituted cytochrome *c*-like compound in the succinate dehydrogenase and cytochrome oxidase systems was 85—90% compared to natural cytochrome *c*.[4] The reaction between (2) and thiol was also used for the preparation of *c*-type haemopeptide or synthetic protein.[5,6] In spite of these successes in forming the sulphide bond, the reaction mechanism remained to be elucidated.

On the other hand the incorporation of ^{14}C-labelled protoporphyrin into cytochrome *c* in yeast [7], and of ^{59}Fe-labelled protohaemin into cytochrome *c* of *P. polycephalum*,[7] have been reported. Therefore, protohaemin (3) rather than (2) was suggested to be a direct precursor of cytochrome *c* in biosynthesis, and accordingly it became imperative to examine what kind of reaction could afford a sulphide bond starting from (3) and cysteine under moderate conditions as described above. In a previous communication we demonstrated that haemin *c* (4) [iron(III) complex of (1)] was prepared in high yields by incubating (3), L-cysteine, and sodium borohydride in the presence of cetyltrimethylammonium bromide (CTAB) micelle and oxygen at pH 8.1 for 3 min.[8] This paper deals with further studies and presents a novel radical mechanism of the addition reaction, in which hydrogen atom generated by the reaction of (3) with sodium borohydride adds to the vinyl group of (3) yielding a stable α-carbon radical, which couples with the thiyl radical forming sulphide linkages at the α-carbon of (3).

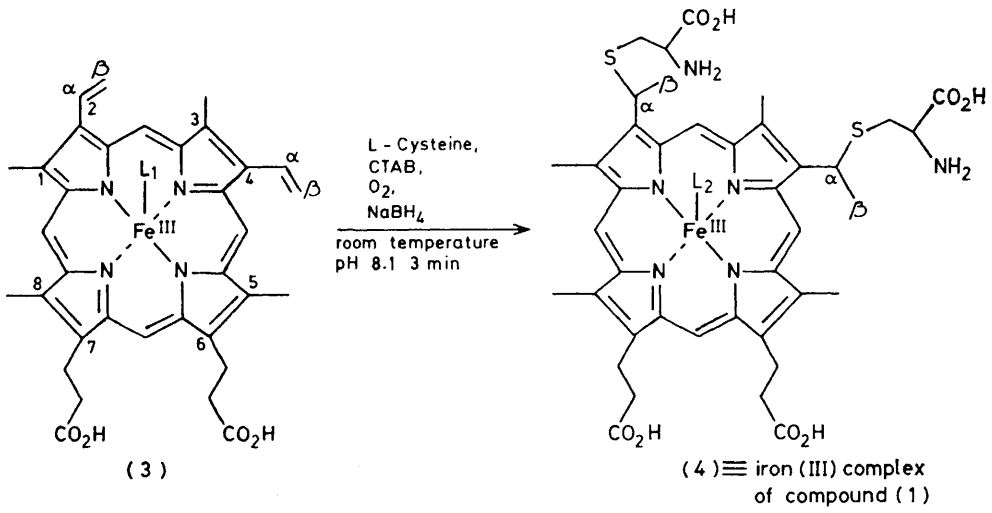
RESULTS AND DISCUSSION

Formation of Haemin c (4) *from Protohaemin* (3) *and* L-*Cysteine.*—As shown in Scheme 1, (4) was prepared in 45—50% yield by the reaction of (3) with L-cysteine and sodium borohydride when a cetyltrimethylammonium bromide (CTAB) micelle in phosphate buffer (0.2M, pH 8.1) and a low concentration of oxygen were provided.

The product was identified as the expected (4) by spectral analyses as well as based on its chemical conversion into the known compounds, haematohaemin (5) [3,6] (95% yield) and haematoporphyrin dimethyl ester dimethyl ether (6) [9] (85% yield) by established procedures. Haemin *c* (4) was also converted into bis-(*N*-benzyloxycarbonyl)porphyrin *c* tetramethyl ester (7), which was identified by n.m.r. and mass spectra. Two cysteic acid residues formed by performic acid oxidation of (4) were determined by amino-acid analysis.

Essential Role of the Reaction Components.—The sulphide bond was not formed under similar conditions when protoporphyrin (8) was used instead of protohaemin (3). Cyanide anion or carbon monoxide completely inhibited the formation of (4) from (3). These results clearly indicate that the iron of (3) is essential for this reaction. No occurrence of the reaction under strictly deoxygenated conditions also indicated an obligatory role of oxygen. Sodium borohydride could not be replaced by other reducing agents such as sodium dithionite, sodium sulphite, sodium ascorbate, cysteine, or diborane; thus the involvement of 'hydride' ion in the initial reducing process is suggested.

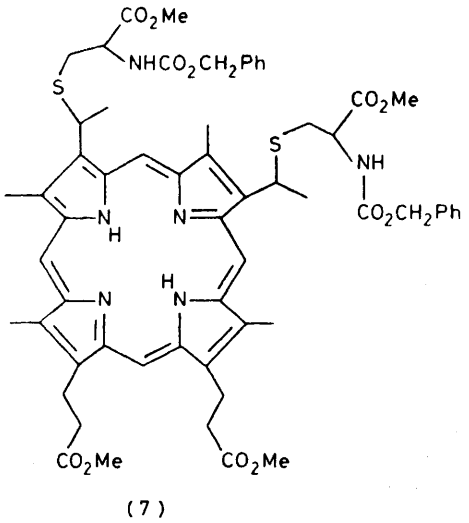
The yield of (4) was low when CTAB was omitted from the reaction mixture or when the reaction was carried out in a neutral (Triton X) or anionic (SDS) micelle. Notably CTAB prevented demetallation from haemins



SCHEME 1

during the borohydride reduction even when borohydride was in a large (*e.g.* 600-fold molar) excess over haemin.

Spectral Changes during the Reaction.—The absorption spectra observed during the reaction are shown in the Figure. Reaction conditions were identical with those of



(7)

the synthetic procedure. The reaction mixture was continuously circulated between an optical cell and a reaction vessel by a small pump through a Teflon tube to follow the course of the reaction. Before addition of cysteine to the reaction mixture, spectrum (*a*) was obtained, in agreement with the reported spectrum of protohaemin (3) in a CTAB micelle.[10] Upon the addition of cysteine, the spectrum immediately changed to (*b*), which suggested the formation of a high-spin iron(III) complex in which cysteine might be co-ordinated to the iron atom. Addition of sodium borohydride then gave an iron(II) type spectrum (*c*), and then an iron(III)-type spectrum (*d*) appeared at the end of the incubation.

When the cysteine was omitted from the reaction mixture the reduction of the Fe^{III} of (3) with sodium borohydride was very slow, and accordingly, the co-ordination of cysteine to the iron atom of (3) was suggested to facilitate the reduction of the Fe^{III}.

Formation of Mesohaemin (9) *and Haematohaemin* (5) *by Reaction of Protohaemin* (3) *with an Excess of Sodium Borohydride.*—When (3) was treated with an excess of sodium borohydride [344 molar excess over (3)] for 3 min in the presence of CTAB and a low concentration of oxygen (see Experimental section) at pH 8.1, mesohaemin (9), haematohaemin (5), and haemins containing a vinyl, ethyl, or hydroxyethyl group at the 2- or 4-position were obtained (Scheme 2). Demetallation scarcely occurred during the reaction [less than 0.5% of (3)]. The products [(9) and (5)] were further converted into the corresponding porphyrin dimethyl ester and dimethyl ester dimethyl ether [(10) and (6) respectively]. The

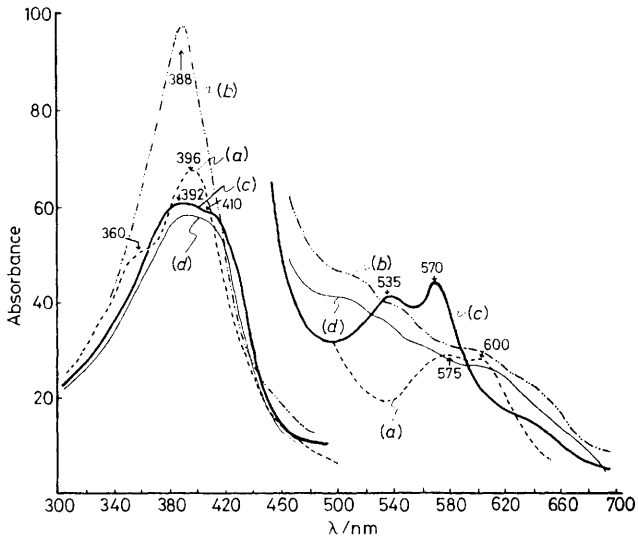
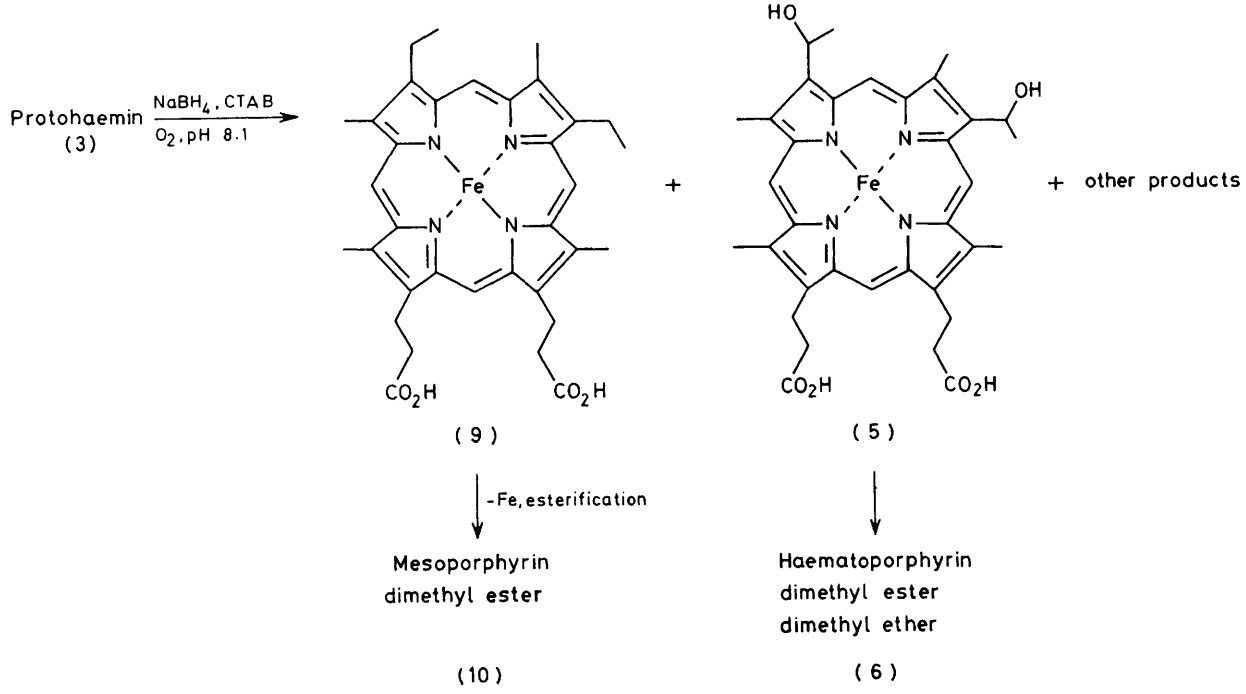


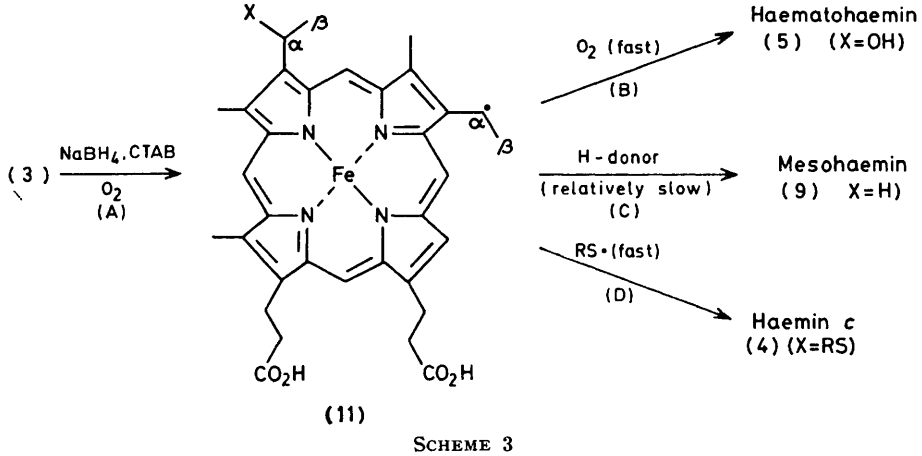
FIGURE Spectral change accompanying the addition of cysteine and $NaBH_4$ to protohaemin-CTAB solution at pH 8.1, 25 °C (for details see text); (*a*) protohaemin (10 mg) in sodium phosphate buffer (200 ml) (0.2M, pH 8.1) containing CTAB (72 mg); oxygen concentration (15 μM) of the solution was maintained by nitrogen bubbling; (*b*) (*a*) + cysteine (500 mg); (*c*) + $NaBH_4$ (20 mg); (*d*) 3 min after (*c*)

yields of purified (10) and (6) were 20 and 4%, respectively. When the reaction was carried out under air to increase the oxygen concentration, the yields of (10) and (6) were 3 and 6%, respectively, and highly autoxidized products of unknown nature predominated. The addition of hydroquinone (10^{-3}M) increased the yield of (6) the relative yields of (5) and (9) would be determined by the competitive action of oxygen and a hydrogen donor on (11), as shown in Scheme 3.

The formation of (5) and (9) from (3) was inhibited completely by cyanide anion or carbon monoxide. Under strictly anaerobic conditions, (3) remained un-



SCHEME 2



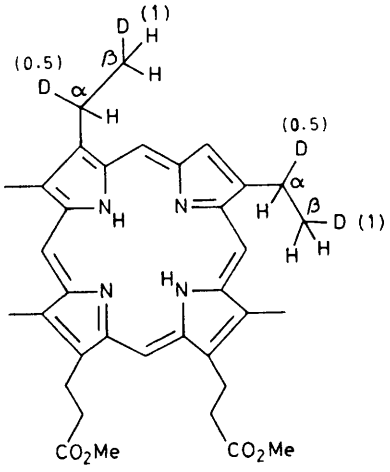
SCHEME 3

up to 17%, possibly preventing further oxidation. However, a large excess of hydroquinone (4×10^{-2}M) inhibited even initiation, and no reaction took place.

The simultaneous transformation of a vinyl group into either an ethyl or hydroxyethyl group, and the fact that the yields of the products [(9) and (5)] are affected by the oxygen concentration strongly suggest that (11), with an α-carbon radical of the 2- or 4-ethyl group [derived from protohaemin (3)], is a common intermediate for mesohaemin (9) and haematohaemin (5). Thus, changed and was recovered almost quantitatively. Also, the vinyl group was not transformed into an ethyl or hydroxyethyl group when protoporphyrin (8) was used as a starting material. These observations, which are common to both reactions represented by Schemes 1 and 2, indicate that the iron of (3) and oxygen are also obligatory in the reaction of Scheme 2, and that they also play an essential role in the formation of the intermediate (11) [step (A), radical-forming process]. Therefore involvement of step (A) and the common inter-

mediate (11) may be postulated in the reaction of Scheme 1, where (11) gives a sulphide bond on combination with a cysteinyl radical [step (D) in Scheme 3].

Protohaemin (3) was treated with sodium borodeuteride instead of sodium borohydride for 3 min, and the resulting mesohaemin was further converted into deuteriated mesoporphyrin dimethyl ester (12). The n.m.r.

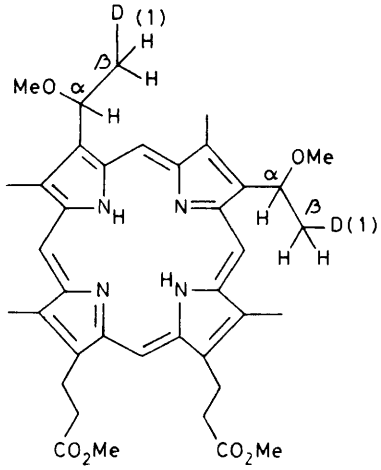


(12)

spectrum of the product confirmed the incorporation of 1 deuterium atom at the β-carbon and 0.5 deuterium atom at the α-carbon of the vinyl group of (3). The incorporation of deuterium at the α-carbon again supports the intermediacy of (11), since borohydride is expected to compete for (11) with other hydrogen-atom donors in the reaction mixture (CTAB and haemins). Another possible candidate for the intermediate may be carbanion formation at the α-carbon, which could be generated formally by hydride (or deuteride) attack at the β-carbon of (3). However, incorporation of deuterium at the α-carbon of the carbanion from sodium borodeuteride is very unlikely to occur in aqueous solution.

Haemin c *Synthesis utilizing Sodium Borodeuteride.*—Haemin *c* was synthesized essentially according to the procedure as summarized in Scheme 1 except that sodium borodeuteride was used instead of sodium borohydride. The haemin *c* thus obtained was converted into haematoporphyrin dimethyl ester dimethyl ether (13) by procedures listed in the literature.[9] The n.m.r. spectrum of (13) indicated the incorporation of the deuterium at the β-carbon of the vinyl group of (3). The incorporation of deuterium and the α-carbon radical (11) formation as suggested above led us to the hypothesis that hydrogen atom is generated by reduction of the iron(III) of (3) with sodium borohydride, followed by its addition to the β-carbon of the vinyl group of (3), resulting in the formation of (11). The intermediate (11) is resonance-stabilized and much more stable than the β-carbon radical so that Markownikoff-type addition does not predominate. Thus the combination of (11) with the cysteinyl radical formed in the reaction mixture is the most probable mechanism for the formation of haemin *c* (4).

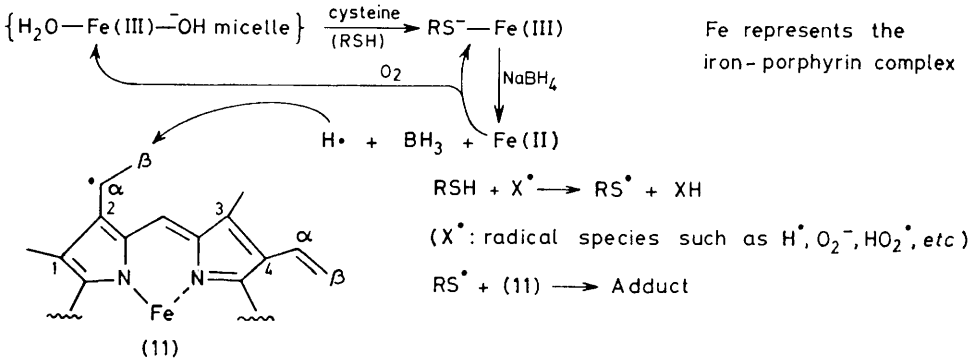
More Evidence for the Generation of Free Hydrogen Atom from (3) *and Sodium Borohydride.*—Protoporphyrin (8) was treated with sodium borohydride in the presence of protohaemin (3), CTAB, and a small amount of oxygen at pH 8 to give a mixture of mesoporphyrin (14), haematoporphyrin (15), and other porphyrins containing a vinyl, ethyl, or hydroxyethyl group at the 2- or 4-position. The products were esterified with methanol and sulphuric acid (95 : 5 v/v) and purified by column



(13)

chromatography. The yield of the main product, mesoporphyrin dimethyl ester (10), was 13%. Seemingly (10) must have been derived from (8), since the removal of iron from the haemins in the reaction mixture scarcely occurred (0.5% at maximum estimation). Mesohaemin (9), derived from protohaemin (3), was converted into (10), accounting for 24% of (3). In the absence of (3), protoporphyrin (8) was not converted into mesoporphyrin (14) or haematoporphyrin (15) with sodium borohydride. This result supports the idea that free hydrogen, produced by the reaction of sodium borohydride with the iron(III) of (3), is transferred to the β-carbon of the vinyl group of (8) to yield the α-carbon radical, and that this radical finally gives (14) or (15).

Mechanism.—Scheme 4 is a proposed mechanism which explains the generation of hydrogen atom followed by its addition to the vinyl group of (3) to form the α-carbon radical (11). The combination of (11) with cysteinyl radical leads to the formation of the sulphide bond. Oxygen is involved not only in the oxidation of iron(II) to iron(III), which produces hydrogen atom by reaction with sodium borohydride, but also in the formation of cysteinyl radical. Other conceivable mechanisms can be eliminated as follows. Acid-catalysed addition of thiol to olefin *via* a carbocation is unlikely, because the present reaction was performed at pH 8.1, and also this mechanism cannot explain the formation of deuterium-incorporated product (13) from sodium borodeuteride.



SCHEME 4 Plausible mechanism for the formation of a Markownikoff-type adduct

Another mechanism to afford a Markownikoff-type adduct might be the generation of sulphenium ion, which could arise *via* a two-electron transfer from cysteinyl anion to oxygen through iron within a ternary complex consisting of haem, oxygen, and cysteinyl anion (O_2–Fe^{II}–$\bar{S}R$). The addition of the resulting sulphenium ion to the double bond of protohaemin (3) would give episulphenium ion, and the ring opening with hydride would result in the formation of haemin *c* (4). Although this mechanism is not excluded completely, the fact that the vinyl group of (3) was efficiently converted into ethyl and hydroxyethyl groups even in the absence of cysteine renders the involvement of the episulphenium ion unlikely.

Although it is of minor importance, a direct reaction of cysteinyl radical with the vinyl group of either haem or haemin is eliminated, since such an addition would lead to an anti-Markownikoff-type adduct.* Even if cysteinyl radical could add to the α-carbon of the vinyl group of haem (or haemin) to form the β-carbon radical, the exclusive incorporation of deuterium into (4) from sodium borodeuteride in the presence of a large excess of a good hydrogen donor (cysteine) is hardly explained.

EXPERIMENTAL

Materials and Methods.—Protohaemin chloride was purchased from Sigma, sodium borohydride from Merck, and sodium borodeuteride from CEA. Authentic samples of mesoporphyrin dimethyl ester [11] and haematoporphyrin dimethyl ester dimethyl ether [12] were prepared according to published procedures. Melting points are uncorrected. Silica gel for column chromatography was Wakogel C-300 (Wako Pure Chemical Ind. Ltd., Osaka, Japan). Visible spectra were recorded on an SM-401 spectrophotometer (Union Giken); n.m.r. spectra on Varian 220 or HA-100D spectrometers (in $CDCl_3$, $SiMe_4$ as internal reference); mass spectra on a Hitachi RMU-6L spectrometer. Field desorption (FD) mass spectra were recorded with a JEOL D3000 spectrometer. The oxygen concentration was determined with a Gilson oxygraph K-1C equipped with an electrode and a Gilson recorder-RP.

Preparation of Haemin c (4) *from Haemin* (3) *and* L-*Cysteine.*—Haemin chloride (3) (10 mg) dissolved in 0.1N sodium hydroxide (10 ml) was added to sodium phosphate buffer (200 ml) (0.2M, pH 8.1) containing CTAB (72 mg, 10^{-3}M) while nitrogen was bubbled through the solution. After the addition of cysteine (500 mg), the oxygen concentration was adjusted to *ca.* 15 μM, sodium borohydride (20 mg) was added, and the reaction mixture was stirred vigorously for another 2 min. The pH of the solution was then adjusted to 3.5 with 6N hydrochloric acid. A small amount of unchanged haemin as well as mesohaemin was removed by extraction with ethyl acetate. The resulting aqueous solution was subjected to column chromatography on Amberlite CG-50 (H^+ form) prepared according to the literature.[3] Elution with pyridine–acetic acid buffer (0.1M, pH 3.8) removed excess of cysteine. After washing of the column with distilled water, the pigment adsorbed on the top of the column was eluted with pyridine–water (1 : 1). The eluate was evaporated under reduced pressure and the residue was dissolved in a small amount of sodium hydroxide solution (0.1N). Haemin *c* (4) was precipitated by the addition of 6N hydrochloric acid, washed with distilled water, and dried over P_2O_5 [yield was 45—50% based on (3)]. The visible spectra of haemin *c* and its pyridine haemochromogen were identical to those reported in the literature.[13]

Preparation of Bis-(N-*benzyloxycarbonyl*)*porphyrin* c *Tetramethyl Ester* (7) *from Protohaemin* (3).—Haemin *c* was prepared according to the procedures described above except that the last re-precipitation was omitted. To crude haemin *c* obtained from eight runs [total amount of starting material (3) was 80 mg], was added methanol (80 ml). After the addition of ferrous sulphate (400 mg), hydrogen chloride gas was introduced into the solution at 0° C. Within 1 min removal of iron from (4) was complete. The reaction mixture was poured into a concentrated solution of Na_2HPO_4, and neutralized with sodium hydrogencarbonate if necessary. The aqueous solution was extracted with butanol (2×) and the resulting butanol layer was washed twice with dilute sodium hydrogencarbonate solution followed by water. After evaporation of the butanol layer, the residue was dissolved in methanol solution (50 ml) containing 20% hydrogen chloride, and the mixture was left for 20 h at room temperature. After evaporation, the residue was dissolved in butanol, the solution was washed several times with dilute aqueous sodium hydrogencarbonate solution and with

* The failure of radical thioylation of protoporphyrin has been reported elsewhere.[1] Our attempted radical addition of *N*-acetyl-L-cysteine or cysteine methyl ester to haemin dimethyl ester in chlorobenzene utilizing an initiator such as azo-isobutyronitrile gave no adduct. The very low reactivity of protoporphyrin and protohaemin toward the thiyl radical may be related to the reversible nature of the addition reaction and the high stability of the α-carbon radical.

water, and then evaporated. To the residue were added chloroform (20 ml) and saturated aqueous sodium hydrogencarbonate (20 ml). After the addition of benzyloxycarbonyl chloride (1.0 ml), the reaction mixture was vigorously stirred at 0° C for 1 h, followed by extraction with ethyl acetate (400 ml). To the extract, pyridine (0.5 ml) was added to decompose any remaining benzyloxycarbonyl chloride. The solution was washed with water, 0.1 N-HCl solution (2×), sodium hydrogencarbonate solution, and then water. Evaporation afforded a residue which was subjected to column chromatography on silica gel utilizing methylene chloride–ethyl acetate (9 : 1) as eluant. After elution of a minor band (presumably monocysteinyl products), compound (7) was eluted out as the main band. Column chromatography was repeated until (7) was pure on the basis of t.l.c. [silica gel, benzene–ethyl acetate (5 : 1) as eluant] to give (7) [38.4 mg, 28% yield based on protohaemin (3) used]; *m/e* (FD) 1 130 (29 5%), 1 129 (50.0), 1 128 (M^+, 78.4), 894 (33.0), 893 (71.6), and 892 (100); δ(60 MHz) 2.35 (d, 6 H), 3.20 (m, 8 H), 3.66 (s, 24 H), 4.4 (m, 6 H), 4.66 and 5.13 (4 H), 5.8 (m, 4 H), 7.35 (s, 10 H), 10.13 (2 H), and 10.56 (2 H); $\lambda_{max.}$ (CH_2Cl_2) 404, 500, 535, 569, 597, and 624 nm.

Reaction of (3) *with Sodium Borohydride at a low Oxygen Concentration*.—Nitrogen was bubbled through a sodium phosphate buffer solution (0.2M, pH 8.1, 400 ml) containing CTAB (144 mg, 10^{-3}M). To the solution was added (3) (40 mg, 61 μmol) dissolved in 0.1N sodium hydroxide solution (10 ml) with stirring. After addition of sodium borohydride (800 mg) dissolved in 0.1N sodium hydroxide solution (10 ml), the reaction mixture was stirred for 3 min with bubbling of nitrogen. The unchanged sodium borohydride was quenched with acetic acid and the solution was adjusted to pH 3.0 with 6N hydrochloric acid. The reaction mixture was extracted ethyl acetate (3×). The combined organic layers were washed with 0.1N hydrochloric acid and dried over sodium sulphate. After evaporation, the residue was dissolved in methanol containing 10% oxalic acid and subjected to the reaction of Grinstein [9a] to remove iron. The chloroform extract was washed with dilute ammonium hydroxide solution and then water, dried over sodium sulphate, and evaporated to dryness. The residue was subjected twice to column chromatography on aluminium oxide eluting with chloroform–methanol (200 : 1 v/v). The eluate was evaporated, the residue dissolved in ethyl acetate, and the ethyl acetate solution washed with water several times. After evaporation, the residue was adsorbed on a silica gel column equilibrated with a benzene–ethyl acetate (5 : 1 v/v). Mesoporphyrin dimethyl ester (10) was eluted at first. This column chromatography was repeated until the purity criteria for (10) and haematoporphyrin dimethyl ester dimethyl ether (6) were satisfied by t.l.c. (silica gel) and visible spectroscopy. The yields of (10) and (6) were 20% and 4%, respectively: (10), m.p. 215—217 °C ($CHCl_3$–MeOH)(lit.,[11] 213—216 °C); $\lambda_{max.}$ ($CHCl_3$) 400, 499, 533, 567, 594, and 621 nm (found to be consistent with the literature [11]); δ (100 MHz) 1.88 (t, 6 H), 3.28 (t, 4 H), 3.67 (s, 18 H), 4.10 (q, 4 H), 4.44 (t, 4 H), and 10.10 (s, 4 H); *m/e* 596 (11.1%), 595 (42.9), and 594 (M^+, 100): (6), $\lambda_{max.}$ (pyridine) 402, 499, 533, 569, and 623 nm (found to be consistent with the literature [12]); δ (100 MHz) 2.25 (d, 6 H), 3.26 (t, 4 H), 3.65 (s, 24 H), 4.38 (t, 4 H), 6.04 (q, 2 H), 10.02, 10.07, 10.48, and 10.53 (all s, 4 H).

Reaction of (3) *with Sodium Borodeuteride at a low Oxygen Concentration*.—The reaction was performed according to the procedure described above except that sodium borodeuteride was used instead of sodium borohydride. A similar treatment gave deuterium-incorporated mesoporphyrin dimethyl ester (12); δ (100 MHz) 1.88 (m, 4 H, CH_2D), 3.24 (t, 4 H), 3.64 (s, 18 H), 4.05 (t-like, 3 H), 4.43 (t, 4 H), and 10.10 (4 H); *m/e* 600 (26.0%), 5.99 (55.2), 5.98 (88.5) 597 (100), 596 (81.3), 595 (22.9), and 594 (3.1).

Deuterium Incorporation into (4).—Haemin *c* was prepared by the method described above utilizing sodium borodeuteride. Deuterium-incorporated haemin *c* was converted into (13) by the established procedures; [9] δ (100 MHz) 2.28 (d, 4 H), 3.34 (t, 3 H), 3.70 (singlets, 24 H), 4.48 (t, 4 H), and 6.10 (t, 2 H).

Reaction of (8) *with Sodium Borohydride in the Presence of* (3), *CTAB, and Oxygen*.—Nitrogen was bubbled through a sodium phosphate buffer solution (0.2M, pH 8.0) (400 ml) containing CTAB (144 mg, 10^{-3}M). To the solution were added (3) (20 mg, 30.7 μmol) in sodium hydroxide solution (0.1N, 10 ml) and (7) (20 mg, 35.5 μmol) in potassium hydroxide solution (0.1N, 10 ml), followed by the addition of sodium borohydride (800 mg) dissolved in 0.1N sodium hydroxide solution (10 ml). The reaction mixture was stirred with bubbling of nitrogen for 3 min, then unchanged sodium borohydride was quenched with acetic acid. The solution was adjusted to pH 3.0 with hydrochloric acid (6N), stirred for 10 min at room temperature under air, and extracted with ethyl acetate (3×). The combined organic layers were washed with water, and porphyrins were extracted with 1N hydrochloric acid. The combined acid solution was adjusted to pH 3 with a saturated sodium acetate solution and extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulphate, and evaporated to dryness. The residue was dissolved in methanol (100 ml) containing sulphuric acid (2.7 ml) and left overnight at room temperature. The reaction mixture was poured into dilute sodium acetate solution, the pH being adjusted to 3.5, followed by extraction with ethyl acetate. The ethyl acetate layer was washed several times with water, dried over sodium sulphate, and then evaporated. The porphyrin esters were chromatographed on aluminium oxide twice, followed by silica gel twice to afford (10) (4.7 μmol, 13%), m.p. 215—217 °C ($CHCl_3$–MeOH)(lit.,[11] 213—216 °C); visible spectrum consistent with the literature; [11] δ (100 MHz) 1.84 (t, 6 H), 3.30 (t, 4 H), 3.84 (s, 18 H), 4.08 (q, 4 H), 4.43 (t, 4 H), and 10.05 (4 H).

The organic layer of the first extract, which contained haemins, was washed with water and evaporated. The residue was subjected to the reaction of Grinstein [9a] for the removal of iron with concurrent esterification. From the mixture, (10) was isolated by column chromatography on aluminium oxide and silica gel in 24% yield, m.p. 215—217 °C ($CHCl_3$–MeOH) (lit.,[11] 213—216 °C); the R_F value on t.l.c. [silica gel, benzene–ethyl acetate (5 : 1) as eluant], visible spectrum, and n.m.r. spectrum were consistent with those of an authentic sample.[11]

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