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Radical Intermediates in the Oxidation of Octaethylheme to Octaethylverdoheme†

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ABSTRACT: Iron(III) oxyoctaethylporphyrin was isolated and purified as a dimer. The addition of tosylmethyl isocyanide to a solution of the dimer produced a monomer species, which was isolated and identified as bis(tosylmethyl isocyanide)iron(II) 5-oxyoctaethylporphyrin π-neutral radical. The product of dissociation of the dimer by imidazole was bis(imidazole)iron(III) 5-oxyoctaethylporphyrin. The spectral properties of the product of dissociation of the dimer by pyridine and published data on bis(pyridine)oxymesoheme and bis(pyridine)oxyprotoheme were consistent with its identification as bis(pyridine)iron(II) 5-oxyoctaethylporphyrin π-neutral radical. When this product was exposed to oxygen, a weak radical signal appeared in its electron spin resonance spectrum, which was attributed to the displacement of one of its pyridine ligands by O_2 to form (pyridine)(dioxygen)iron(II) 5-oxyoctaethylporphyrin π-neutral radical. The pyridine oxygen radical converted spontaneously to octaethylverdohemochrome, which was purified and identified as bis(tosylmethyl isocyanide)iron(II) octaethylverdohemochrome hydroxide. The yield of verdohemochrome from iron oxyporphyrin was increased by the addition of phenylhydrazine or ascorbate. A scheme for the oxidation of iron(III) oxyporphyrin to iron(II) verdoheme by O_2 that proposes a mechanism for the expulsion of CO and the replacement of a methene bridge of the porphyrin ring by an oxa bridge is presented.

An intermediate product in the oxidation of pyridine protohemochrome to pyridine protoverdohemochrome by O_2 in the presence of ascorbic acid was characterized as an iron(III) hematin of an oxyporphyrin (Lemberg et al., 1937, 1938). Exposure of oxymesohemin IXβ dimethyl ester to O_2 resulted in mesoverdohemin dimethyl ester, an iron(III) compound (Jackson et al., 1968). Sano et al. (1981) reported that the electron spin resonance (ESR) spectrum of a mixture of iron oxymesoporphyrin isomers in alkaline pyridine solution at 77 K was characteristic of a high-spin iron(III) compound. When,

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however, the solution was brought to pH 9.5, the resulting ESR spectrum was considered to be that of a low-spin iron(I) compound, and the conversion of iron oxymesoporphyrin to mesoverdohemochrome by O_2 was concluded to involve the oxidation of iron(I) to iron(II). On the other hand, the Mössbauer spectra of iron oxymesoporphyrin resembled those of iron(II) compounds. Recently Sano et al. (1986) concluded that oxyprotohemin IXα in pyridine at room temperature is best described as Fe(II) π-neutral radical mixed with the Fe(I) species. The product of the addition of 2-methylimidazole to an isomeric mixture of iron(III) oxymesoporphyrins in alkaline aqueous ethanol was reported to be the 2-methylimidazole complex of iron(II) oxymesoporphyrin (Sano & Sugiura, 1982). Analytically and isomerically pure pyridine protoverdohemochrome IXα, prepared by the coupled oxidation of myoglobin and ascorbic acid, was determined to be an iron(II) compound (Saito & Itano, 1982), and bis(pyridine)octaethylverdohemochrome was also shown to be in the iron(II) state (Lagarias, 1982; Hirota & Itano, 1983).

For many years, the conversion of oxyheme to verdoheme by O_2 was regarded as an integral step in heme degradation; however, the relevance of this step was questioned when $^{18}O_2$ incorporation studies of the oxidative degradation of heme to biliverdin seemed to indicate that verdoheme was not involved in the process (Tenhunen et al., 1972; Jackson et al., 1978). Recent studies have shown that verdoheme is a valid precursor of biliverdin (Saito & Itano, 1982; Hirota & Itano, 1983; Itano & Hirota, 1985). Renewed investigations of oxyheme and of its conversion to verdoheme seemed appropriate. While this paper was in preparation, Sano et al. (1986) reported studies on the conversion of iron(III) oxyprotoporphyrin IXα to iron biliverdin IXα. Our results with the use of derivatives of octaethylheme agree in general with theirs but differ in postulated structures of intermediates and in details of the proposed mechanism of conversion of oxyheme to verdoheme.

When an asymmetrically substituted heme is oxidized nonenzymically, the product is a mixture of isomers because the four meso positions of the porphyrin ring are oxidized nonspecifically (O'Carra & Colleran, 1970). Octaethylhemin (**1a**) (see Chart I), a symmetrical analogue of heme, has been used as a model compound in order to avoid this complication. Bonnett and Dimsdale (1972) reported the formation of iron(III) oxyoctaethylporphyrin by the reaction of H_2O_2 with pyridine octaethylhemochrome (**1b**) and used this reaction in their preparation of aquairon(III) oxyoctaethylporphyrin (**2a**). The conversion of **2a** to octaethylverdohemochrome (**3a**) by oxidation with O_2 was demonstrated spectrophotometrically. Octaethylverdohemochrome has been synthesized and its structure determined (Lagarias, 1982; Hirota & Itano, 1983), and the mechanism of conversion of octaethylverdohemochrome to octaethylbiliverdin has been elucidated (Hirota & Itano, 1983; Itano & Hirota, 1985). Investigations on the conversion of octaethylhemin to octaethylverdohemochrome via iron oxyoctaethylporphyrin are reported here.

Materials and Methods

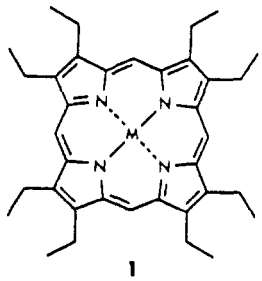
Pyridine was purified by distillation in the presence of ninhydrin. Tosylmethyl isocyanide ($TosCH_2NC$) was purified by column chromatography on neutral alumina (Hoogenboom et al., 1977). Uniplates (silica gel G, Analtech) were used for analytical and preparative thin-layer chromatography (TLC). Octaethylhemin (**1a**), bis(pyridine)iron(II) octaethylporphyrin (**1b**), and 5-oxyoctaethylporphyrin were prepared from octaethylporphine (Porphyrin Products, Logan, UT) according to the procedures of Bonnett and Dimsdale (1972). The other chemicals were purchased from Aldrich (Milwaukee, WI). All syntheses were carried out under nitrogen. Electronic absorption spectra were recorded on a Cary Model 17 or a Hitachi 557 spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer 1330 or a JASCO IRA-1 infrared spectrometer. Proton NMR spectra with internal tetramethylsilane were recorded with a Varian EM 390 (90 MHz) or a modified Varian (360 MHz). Magnetic susceptibility in solution was taken according to the method of Evans (1959) with tetramethylsilane as the standard compound. Magnetic susceptibility in the solid state was recorded on a Model 905 variable-temperature superconducting susceptimeter–magnetometer system. ESR spectra were taken with a Varian E-3 spectrometer or a JEOL JES-FE1XG spectrometer equipped with a variable-temperature accessory and calibrated with 2,2-diphenyl-1-picrylhydrazyl. Fast atom bombardment (FAB) mass spectra were taken at the Midwest Center for Mass Spectrometry, Department of Chemistry, University of Nebraska, by K. B. Tomer. Samples for FAB were dissolved in *o*-nitrophenyl octyl ether and chloroform. Elemental analyses were performed by T. Tashnian, Micro Lab, Department of Chemistry, University of California, Berkeley.

Synthesis of Iron(III) 5-Benzoyloxyoctaethylporphyrin Chloride (**4**). *(i) Oxidation of Bis(pyridine)iron(II) Octaethylporphyrin* (**1b**). A solution of 550 mg (0.74 mmol) of **1b** in 200 mL of N_2-saturated pyridine was heated to 60 °C, and 3.32 mL (0.97 mmol) of 1% H_2O_2 in pyridine was added. The reaction mixture was kept at 60 °C for 15 min, after which time 13 mL of benzoyl chloride was added and allowed to react for 30 min at room temperature (Bonnett & Dimsdale, 1972). The reaction mixture was then evaporated in a vacuum, and the residue was dissolved in 100 mL of $CHCl_3$. The resulting solution was washed with a saturated aqueous solution of $NaHCO_3$ (2 times) and H_2O (2 times), dried over Na_2SO_4, filtered, and evaporated. The residue was applied to a silica gel column and eluted with $CHCl_3$, 1% methanol in $CHCl_3$, 2% methanol in $CHCl_3$, and 4% methanol in $CHCl_3$ (v/v). The eluates were washed with 1 M HCl and H_2O. After recrystallization from $CHCl_3$ and *n*-hexane, 398 mg (0.54 mmol, $Y = 73\%$) of **4** was obtained. Octaethylhemin (**1a**) (110 mg, 0.18 mmol, $Y = 24\%$) was recovered. A sample of **4** for elemental analysis was dried at 110 °C for 12 h. Anal. Calcd for $C_{43}H_{48}N_4FeO_2Cl$: C, 69.40; H, 6.50; N, 7.53; Fe, 7.50; Cl, 4.76. Found: C, 69.17; H, 6.48; N, 7.53; Fe, 7.50; Cl, 5.34. IR (KBr) 1740 cm^{-1}; λ_{max}^{nm} (ϵ_{mM}) ($CHCl_3$) 383 (75.8), 505 (7.49), 533 (6.30), 638 (3.50).

(ii) Coupled Oxidation of Octaethylhemin (**1a**). The hemin **1a** (104 mg, 0.17 mmol) was dissolved in 200 mL of pyridine/water (4:1 v/v) in a round-bottom flask (500 mL). After N_2 gas was passed through the mixture for more than 15 min, the flask was evacuated, and about 60 mL of oxygen and 131 mg (0.91 mmol) of phenylhydrazine hydrochloride ($PhNHNH_2$) were added and allowed to react at room temperature for 10 min (the color changed from black to red to green; the visible spectrum was checked). Benzoyl chloride (2 mL) was then added and allowed to react for 30 min. The reaction mixture was evaporated, and the residue was treated by the procedure described above to obtain 38.5 mg (50 μmol, $Y = 30\%$) of **4** and recover 14.1 mg (23 μmol, $Y = 14\%$) of **1a**.

Reduction of **4**. Compound **4** (49.5 mg, 66.6 μmol) was dissolved in 10 mL of pyridine, and 0.25 mL of 95% hydrazine was added. The reaction mixture was heated at 50 °C for 5 min and cooled, and 0.4 mL of AcOH and 15 mL of H_2O were then added. After 30 min at 4 °C, crystals were filtered, washed twice with H_2O, and dried to obtain 48.5 mg (60.2

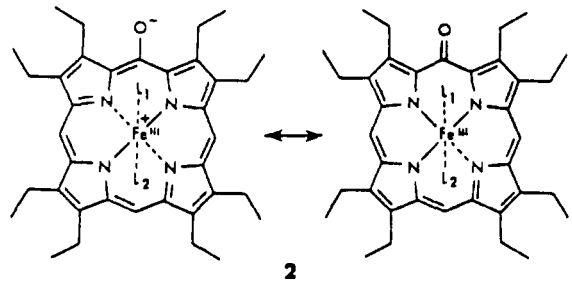
Chart I



1

a M = $Fe^{III}Cl$

b M = $Fe^{II}(C_5H_5N)_2$

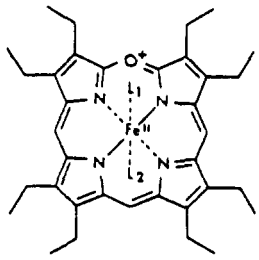


2

a L_1 = H_2O

b L_1 = L_2 = Imidazole

c L_1 = L_2 = C_5H_5N

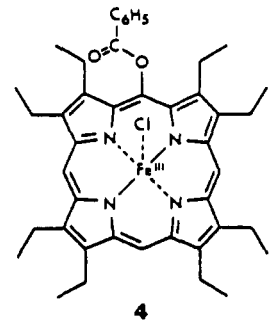


3

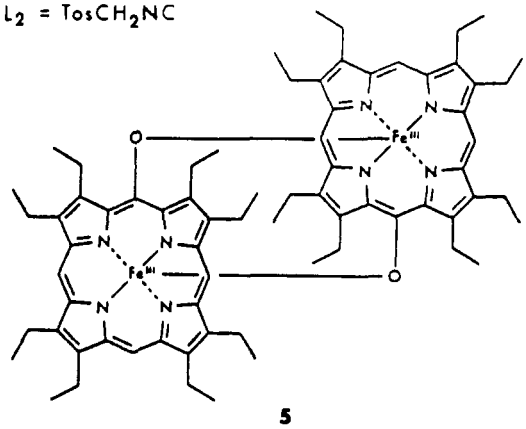
a L_1 = L_2 = C_5H_5N

b L_1 = L_2 = $TosCH_2NC$

c L_1 = C_5H_5N, L_2 = $TosCH_2NC$



4



5

μmol, Y = 90%) of (pyridine)iron(II) 5-benzoyloxyoctaethylporphyrin: λ_{max}^{nm} (ϵ_{mM}) ($CHCl_3$) 410 (120), 480 (10.6, sh), 510 (15.1, sh), 519 (17.1), 546 (19.9); IR (KBr) 1730 cm^{-1}; NMR (C_5D_5N) δ 1.80 (m, 24 H), 3.90 (m, 16 H), 7.17 (m, 4 H, β-H), 7.50 (m, 3 H, *m*- and *p*-H), 7.80 (m, 2 H, γ-H), 8.71 (m, 4 H, α-H), 8.97 (m, 2 H, *o*-H), 9.93 (s, 2 H, 10- and 20-H), 10.05 (s, 1 H, 15-H). The sample for elemental analysis was dried at room temperature for 48 h. Anal. Calcd for $C_{43}H_{48}N_4FeO_2 \cdot C_5H_5N \cdot H_2O$: C, 71.54; H, 6.88; N, 8.69; Fe, 6.93. Found: C, 71.32; H, 6.74; N, 8.47; Fe, 7.09.

Demetalation of **4**. Compound **4** (21.0 mg, 28 μmol) was dissolved in pyridine and demetalated by the ferrous sulfate method (Fuhrhop & Smith, 1975). 5-Benzoyloxyoctaethylporphyrin (Bonnett et al., 1969) (12.0 mg, 18 μmol, Y = 65%) was obtained.

Synthesis of Iron(III) 5-Oxyoctaethylporphyrin Dimer (**5**). (*i*) *Methanolysis of* **4**. Compound **4** (214 mg, 0.288 mmol) in 4 mL of pyridine was added to 40 mL of 1 M $NaOCH_3$ in methanol under N_2. The reaction mixture was allowed to stand at room temperature for 5 h, after which time 200 mL of H_2O was added. Crystals were washed twice with water and dried over $CaCl_2$ in vacuo. A solution of these crystals in $CHCl_3$ was applied to a silica gel column (2 × 13 cm) and eluted successively with $CHCl_3$ and 1% MeOH in $CHCl_3$. $CHCl_3$ without added MeOH eluted the dimer (**5**). Recrystallization from $CHCl_3$ and *n*-hexane gave 88 mg (70 μmol, Y = 49%) of **5**. These purification procedures were done as rapidly as possible because the product gradually decomposed even in $CHCl_3$. Elemental analyses of **5** were taken several times: (i) A sample was dried at room temperature under vacuum for 48 h. Anal. Calcd for $C_{72}H_{86}N_8Fe_2O_2 \cdot 2^2/_3H_2O$: C, 68.89; H, 7.33; N, 8.93; Fe, 8.90. Found: C, 68.87; H, 7.33; N, 8.81; Fe, 8.80. (ii) A sample was dried at 110 °C under vacuum for 16 h. Anal. Calcd for $C_{72}H_{86}N_8Fe_2O_2 \cdot H_2O$:

C, 70.58; H, 7.24; N, 9.15; Fe, 9.12. Found: C, 70.71; H, 7.22; N, 9.11; Fe, 9.03. (iii) A sample was dried at 170 °C under vacuum for 24 h. Anal. Calcd for $C_{72}H_{86}N_8Fe_2O_2 \cdot {}^1/_3H_2O$: C, 71.28; H, 7.20; N, 9.24; Fe, 9.21. Found: C, 71.21; H, 7.07; N, 9.31; Fe, 9.15. IR (KBr), no carbonyl band; λ_{max}^{nm} (ϵ_{mM}) ($CHCl_3$) 390 (124), 490 (31.1), 530 (25.6), 670 (5.90), 1050 (20.5). For a dimer (**5**) with two iron atoms, the magnetic susceptibility per iron in solid showed $\mu_{eff} = 4.78\ \mu_B$ (at 299.8 K) and $\mu_{eff} = 2.91\ \mu_B$ (at 77.1 K). The variation of μ_{eff} with temperature (6.0–299.8 K) gave good agreement with the theoretical curve for a spin-coupled ($^5/_2$, $^5/_2$) system (Earnshaw & Lewis, 1961) with $J = -12\ cm^{-1}$ and $g = 2.0$. The magnetic susceptibility in $CDCl_3$ was $\mu_{eff} = 4.8\ \mu_B$ (at 295 K) per iron. ESR (in $CHCl_3$) showed no signal at 295 K and weak signals that could not be characterized at 77 K; $g_\perp = 5.7$ and $g_\parallel = 1.8$ at 4.8 K. FAB mass spectrum: *m/e* (%) 1207 (70), 1206 (M^+, 31), 690 (21), 631 (21), 605 (31), 604 (41), 603 ($M^+/2$, 100), 602 (47), 572 (39). High-resolution mass spectrum at *m/e* 603 showed 603.2785 (603.2785 was calculated for $C_{36}H_{43}N_4FeO$).

(ii) Insertion of Iron into 5-Oxyoctaethylporphyrin. 5-Oxyoctaethylporphyrin (40.3 mg, 73 μmol) was allowed to react with $FeCl_3$ in AcOH under N_2 gas. Crystals were filtered and washed with an aqueous solution saturated with $NaHCO_3$ and then with H_2O and dried. These crystals (29 mg) showed almost the same spectral data as those reported by Bonnett and Dimsdale (1972) for **2a**. However, the product was found to be a mixture by TLC (0.25 mm) when developed with $CHCl_3$. The major spot ($R_f = 0.8$) was that of the dimer (**5**), and no spot corresponding to monomer was detected. The crystals were dissolved in a small amount of $CHCl_3$ and purified by silica gel column (2 × 13 cm) chromatography as described above. The dimer (**5**) (8.9 mg, 7.4 μmol, $Y = 20\%$) and octaethylbiliverdin (2.0 mg, 3.7 μmol, $Y = 5\%$) were obtained.

Demetalation of **5**. Compound **5** (43.2 mg, 35.8 μmol) was dissolved in pyridine, demetalated by the ferrous sulfate method, and purified with a silica gel column to obtain 30.4 mg (55.2 μmol, $Y = 77\%$) of 5-oxyoctaethylporphyrin: NMR (240 mM NH_2NH_2 in C_5D_5N) δ 1.55 (m, 24 H), 3.33 (q, $J = 7.5$ Hz, 12 H), 3.85 (q, $J = 7.5$ Hz, 4 H), 7.90 (s, 1 H), 8.53 (s, 2 H).

Benzoylation of **5**. To 24.9 mg (20.6 μmol) of **5** in pyridine (5 mL) under N_2 gas, 1 mL of benzoyl chloride was added and allowed to react for 30 min at room temperature. The reaction mixture was then diluted with $CHCl_3$, washed with an aqueous solution saturated with $NaHCO_3$, washed with H_2O, dried, and evaporated. The residue was purified on a silica gel column and converted to the chloride to obtain 30.0 mg (40.3 μmol, $Y = 98\%$) of iron(III) 5-benzoyloxyoctaethylporphyrin chloride (**4**).

Reduction of **5**. Bis(pyridine)iron(II) 5-oxyoctaethylporphyrin was obtained by reacting **5** with hydrazine in pyridine, but its instability in oxygen precluded its isolation as crystals. NMR (250 mM NH_2NH_2 in C_5D_5N) δ 1.88 (m, 24 H), 3.92 (q, $J = 7.5$ Hz, 12 H), 4.28 (m, 4 H), 9.75 (s, 1 H), 9.83 (s, 2 H); IR (2.5% NH_2NH_2 in $CHCl_3$), no carbonyl band; λ_{max}^{nm} (ϵ_{mM}) (1 mM NH_2NH_2 in C_5H_5N) 418 (154), 519 (22.2), 547 (15.3).

Dissociation of **5** *to Monomer. (i) Synthesis of Bis(tosylmethyl isocyanide)iron(II) 5-Oxyoctaethylporphyrin Radical* (**6a**). Tosylmethyl isocyanide ($TosCH_2NC$; 18.0 mg, 92.3 μmol) was dissolved in 2 mL of $CHCl_3$ under N_2 gas, and 15.0 mg (12.3 μmol) of **5** was added to the solution. The reaction mixture was allowed to stand at room temperature for 1 h. After 40 mL of *n*-hexane was added, crystals were collected, washed with *n*-hexane, and dried at room temperature for 24 h to obtain 13.0 mg (10.5 μmol, $Y = 85\%$) of **6a**. Anal. Calcd for $C_{36}H_{43}N_4FeO \cdot (C_9H_9NO_2S)_3 \cdot 3H_2O$: C, 60.86; H, 6.16; N, 7.89; Fe, 4.49. Found: C, 60.99; H, 5.88; N, 8.05; Fe, 4.38. This sample was not purified further because of its instability. IR (KBr) 1530 cm^{-1}; λ_{max}^{nm} (ϵ_{mM}) (45 mM $TosCH_2NC$ in $CHCl_3$) 400 (46.9, sh), 423 (82.6), 531 (7.03, sh), 620 (3.05, sh), 812 (3.52); NMR (150 mM $TosCH_2NC$ in $CDCl_3$) δ 1.4 (br s, 24 H), 7.3 (br s, 16 H) (signals of the meso protons were not detected); ESR (45 mM $TosCH_2NC$ in $CHCl_3$) $g = 2.012$ (peak to peak width = 12 G) at 295 K. The yield of **6a** from **5** was estimated to be 98% from the intensity of the signal. Measurement of the magnetic susceptibility of **6a** (45 mM $TosCH_2NC$ in $CDCl_3$) showed $\mu_{eff} = 1.8\ \mu_B$ (for a radical, spin-only $\mu = 1.73\ \mu_B$). FAB mass spectrum: *m/e* (%) 1207 (1), 1206 (2), 995 (2), 994 (3), 993 (M^+, 5), 798 ($M^+ - TosCH_2NC$, 3), 760 (7), 759 (18), 758 (25), 605 (38), 604 (86), 603 ($M^+ - 2TosCH_2NC$, 100), 602 (67), 601 (33). When 0.4 mL of 0.5 M $TosCH_2NC$ was added anaerobically to 4 mL of a solution of **5** in $CHCl_3$ (ca. 8 μM), complete dissociation to **6a** took place in 0.5 h. Electronic spectra of this dissociation process showed six isosbestic points (Figure 1). The monomer **6a** slowly changed to an unidentified compound; about 40% of **6a** was converted after 38 h.

(ii) Dissociation to Bis(pyridine)iron(II) 5-Oxyoctaethylporphyrin Radical (**6b**). The dissociation of **5** in $CHCl_3$ was complete in concentrations of pyridine higher than 0.62 M. Electronic spectra of this process showed five isosbestic points (Figure 1). Titration (Lemberg & Legge, 1949) showed that two pyridine molecules were bound per iron porphyrin. IR (C_5H_5N) 1540 cm^{-1}; λ_{max}^{nm} (ϵ_{mM}) (0.62 M C_5H_5N in $CHCl_3$) 424 (79.0), 526 (6.70), 604 (6.99, sh), 640 (7.24). Its magnetic susceptibility in 0.62 M C_5D_5N in $CDCl_3$ was $\mu_{eff} = 1.8\ \mu_B$. ESR (0.62 M C_5H_5N in $CHCl_3$) showed no radical signal at 295 K and $g_\perp = 2.37$ and $g_\parallel = 1.71$ at 103 K. FAB mass spectrum: *m/e* (%) 603 ($M - 2C_5H_5N$, 100). The structure of this product will be discussed below. When **6b** was exposed to oxygen, a weak radical signal ($g = 2.008$, peak to peak width = 12 G) was detected at 293 K. Its intensity was about one-hundredth the concentration of the monomer (**6b**), and it was assigned to the (pyridine)(dioxygen)iron(II) oxyoctaethylporphyrin radical (**6c**).

(iii) Dissociation to (Pyridine)(carbon monoxy)iron(II) 5-Oxyoctaethylporphyrin Radical (**6d**). Spectra were taken of a solution of **5** in CO-saturated pyridine. ESR (C_5H_5N saturated with CO) showed $g = 2.008$ (peak to peak width = 10 G) at 293 K. The intensity of the signal corresponded to 66% of the concentration of monomer **6d**. λ_{max}^{nm} (ϵ_{mM}) (C_5H_5N saturated with CO) 416 (61.3), 527 (6.03), 608 (4.02), 650 (3.73), 792 (3.90); IR (C_5H_5N saturated with CO) 1980, 1540 cm^{-1}.

(iv) Dissociation to (Pyridine)(tosylmethyl isocyanide)iron(II) 5-Oxyoctaethylporphyrin (**6e**). To **6b** in pyridine, 1 M $TosCH_2NC$ in pyridine was added under N_2 gas. Formation of **6e** was complete when the concentration of $TosCH_2NC$ was higher than 31 mM. λ_{max}^{nm} (ϵ_{mM}) (31 mM $TosCH_2NC$ in pyridine) 400 (57.7), 423 (102), 550 (7.37, sh), 615 (6.01, sh), 830 (2.91). ESR (59 mM $TosCH_2NC$ in $CHCl_3$) showed $g = 2.012$ (peak to peak width = 12 G) at 293 K. Its intensity corresponded to 3% of the concentration of monomer **6e**.

(v) Dissociation to Bis(imidazole)iron(III) 5-Oxyoctaethylporphyrin (**2b**). To a solution of **5** in $CHCl_3$ was added 1 M imidazole in $CHCl_3$ under strictly anaerobic conditions.

Dissociation of **5** was complete at concentrations of imidazole higher than 62.5 mM. ESR (170 mM imidazole in $CHCl_3$) showed no signal at 293 K and $g_1 = 2.86$, $g_2 = 2.29$, and $g_3 = 1.53$ at 77 K. λ_{max}^{nm} (ϵ_{mM}) (62.5 mM imidazole in $CHCl_3$) 422 (89.1), 528 (7.13), 626 (8.71), 660 (8.08, sh); IR (200 mM imidazole in $CHCl_3$), no carbonyl band.

Compounds **6b**, **6d**, **6e**, and **2b** were less stable than **6a**. They reacted rapidly with oxygen to produce **3**, so that their isolation as crystals was not possible.

Conversion of **5** *to* **3**. (*i*) *Synthesis of Bis(tosylmethyl isocyanide)iron(II) Octaethylverdohemochrome Hydroxide* (**3b**). Iron(II) octaethylverdoheme has been isolated and characterized as the bis(pyridine) tetrafluoroborate (Lagarias, 1982), the bis(pyridine) chloride (Hirota & Itano, 1983), and the bis(tosylmethyl isocyanide) tetrafluoroborate (Saito & Itano, 1986). It is more stable and more readily purified as the bis(tosylmethyl isocyanide) complex (**3b**) than as the bis(pyridine) complex (**3a**). Compound **5** (15.0 mg, 12 μmol) in a 100-mL flask was dissolved in 20 mL of pyridine saturated with N_2 gas. The flask was alternately evacuated by water pump and filled with N_2 gas 3 times. After the fourth evacuation, 20 mL of oxygen was added and allowed to react for 1.5 h. $TosCH_2NC$ (36 mg) was added, and the reaction mixture was evaporated. The residue was dissolved with a small amount of $CHCl_3$ and applied to a TLC plate (200 × 200 × 1 mm), developed with 10% MeOH in $CHCl_3$, scraped off, and extracted, filtered, and evaporated to obtain 14.0 mg (13 μmol, $Y = 53\%$) of **3b**. It was crystallized from $CHCl_3$ and *n*-hexane, mp 156–159 °C. Elemental analysis was performed after the sample was dried at room temperature under vacuum for 48 h. Anal. Calcd for $C_{53}H_{62}N_6FeO_6S_2 \cdot {}^2/_3CHCl_3$: C, 59.76; H, 5.86; N, 7.79; Fe, 5.18. Found: C, 59.45; H, 5.75; N, 7.69; Fe, 4.66. IR ($CHCl_3$) 2140, 1335, 1150, 1010 cm^{-1}. FAB mass spectrum: *m/e* (%) 982 (2), 981 ($M^+ - OH$, 2), 593 (13), 592 (45), 591 ($M^+ - 2TosCH_2NC - OH$, 100), 590 (25). When $^{18}O_2$ was used instead of $^{16}O_2$, the mass spectrum showed that all of **3b** contained ^{18}O (*m/e* 593). λ_{max}^{nm} (ϵ_{mM}) (C_5H_5N) 386 (44.0), 440 (12.0, sh), 495 (6.06), 526 (10.3), 609 (10.0, sh), 651 (33.3); NMR ($CDCl_3$) δ 1.65 (m, 24 H), 2.30 (s, 6 H), 3.50 (m, 20 H), 6.56 (d, $J = 8.4$ Hz, 4 H), 7.00 (d, $J = 8.4$ Hz, 4 H), 8.92 (s, 1 H), 9.05 (s, 2 H); NMR (C_5D_5N) δ 1.63 (m, 24 H), *2.20* (s, 3 H), 2.26 (s, 3 H), 3.50 (m, 16 H), 3.90 (s, 2 H), *5.91* (s, 2 H), 6.75 (d, $J = 8.4$ Hz, 2 H), 7.09 (d, $J = 8.4$ Hz, 2 H), *7.29* (d, $J = 8.4$ Hz, 2 H), *8.12* (d, $J = 8.4$ Hz, 2 H), 9.23 (s, 1 H), 9.61 (s, 2 H). The italicized signals were assigned to the protons of free $TosCH_2NC$. When **3b** was dissolved in C_5D_5N, one of the ligands was displaced by pyridine, and (pyridine)(tosylmethyl isocyanide)iron(II) octaethylverdohemochrome (**3c**) apparently was formed. A spectrum nearly the same as that of **3b** in $CHCl_3$ was obtained when an excess of $TosCH_2NC$ was added to a pyridine solution of **3b**. A solution of **3b** in pyridine was applied to a TLC plate developed with C_6H_6/C_5H_5N/ethanol (14:5:1 v/v) and purified. The NMR spectrum (C_5D_5N) showed a mixture of **3a** and **3c**.

(*ii*) *Spin-Trapping Experiment with DMPO* (*5,5-Dimethyl-1-pyrroline N-Oxide*). To an ESR tube (i.d. 1.3 mm) that contained 0.15 mL of 0.13 mM **5** in $CHCl_3$ were added 50 μL of pyridine and 50 μL of 150 mM DMPO in $CHCl_3$ under N_2 gas. At first no signal was detected, but signals ($g = 2.006$, $a^N = 14.2$ G, and $a^H = 11.9$ G) gradually appeared following exposure to oxygen. These signals, which were distinguished from those of the superoxide radical adduct in that they showed no other splitting even if the modulation of the ESR was adjusted to less than 0.5 G, were assigned to the DMPO adduct of **7**. The signal intensity corresponded to about 4.3% of the concentration of **6b**.

(*iii*) *Effect of Addition of Phenylhydrazine or Ascorbic Acid on the Reaction of* **6b** *with Oxygen*. To 4 mL of about 6 μM **5** in $CHCl_3$ were added simultaneously 0.2 mL of pyridine and 20 μL of 100 mM phenylhydrazine hydrochloride in H_2O (or 10 μL of 20 mM ascorbic acid) in the presence of oxygen. The yields of **3a** were calculated from visible spectra before and after the reaction. The concentration of **3a** was estimated from the absorbance at 652 nm (Hirota & Itano, 1983) and that of **5** from the absorbance at 390 nm. When phenylhydrazine or ascorbate was added, the yield was estimated to be 80%; in the absence of added reducing agent the yield was 47%.

Results and Discussion

In an attempt to prepare iron(III) 5-oxyoctaethylporphyrin (**2a**) as described by Bonnett and Dimsdale (1972), 5-oxyoctaethylporphyrin was treated with $FeCl_3$. Instead of **2a**, however, a different product (compound A), which had no carbonyl group, was obtained in low yield. A different approach to the preparation of **2a** was therefore taken. When bis(pyridine)iron(II) octaethylporphyrin (**1b**) was oxidized with H_2O_2 and benzoylated, iron(III) 5-benzoyloxyoctaethylporphyrin (**4**) was obtained in good yield. Compound **4** was also prepared by the coupled oxidation in aqeous pyridine of octaethylhemin (**1a**) and phenylhydrazine with oxygen followed by treatment with benzoyl chloride. The structure of **4** was supported by spectral data and elemental analysis and by the following reactions. When **4** was reduced with hydrazine in pyridine, (pyridine)iron(II) 5-benzoyloxyoctaethylporphyrin was obtained. Demetalation of **4** with $FeSO_4$ (Fuhrhop & Smith, 1975) gave 5-benzoyloxyoctaethylporphyrin in fair yield. When **4** was treated with 1 M sodium methoxide in methanol in an attempt to prepare **2a**, compound A, a previously unreported compound, was obtained in good yield.

Three reactions were used to elucidate the structure of **A**. (i) By the demetalation of compound A, 5-oxyoctaethylporphyrin was isolated in excellent yield. (ii) By benzoylation of compound A, iron(III) 5-benzoyloxyoctaethylporphyrin chloride (**4**) was isolated from the reaction of compound A with benzoyl chloride in pyridine. (iii) By reduction of compound A, bis(pyridine)iron(II) 5-oxyoctaethylporphyrin was obtained from the reaction of compound A with hydrazine in pyridine. Compound **2** would have yielded the same products in these reactions and might have been isolated as the methanolic adduct inasmuch as methanolic NaOMe was used in the conversion of **4** to compound A. Slow dissociation of ligand as well as slow dissociation of dimer could have accounted for the slowness of conversion of compound A to compound B (Figure 1). However, compound A was also obtained from the insertion of iron into 5-oxyoctaethylporphyrin by a procedure in which methanol was not used. Elemental analyses for compound A dried under vacuum at different temperatures and time spans were in excellent agreement with compositions calculated for **5** with $2^2/_3$, 1, and $^1/_3H_2O$. Almost as good agreement was obtained with compositions calculated for $1^1/_2$, $^1/_2$, and $^1/_6$ CH_3OH, respectively, per molecule of **2**. However, analyses with only $^1/_2$ and $^1/_6$ CH_3OH and the absence of a peak at *m/e* 635 in the FAB spectrum argued against a methanolic adduct of **2**. A μ-oxo dimer of **2** was excluded by the analysis that showed $^1/_3$ H_2O per dimer and by the magnetic moment per iron of **5**, which was significantly higher than that reported for μ-oxo dimers of iron(III) porphyrins (Cohen, 1969; Fleischer & Srivastava, 1969; Moss et al.,

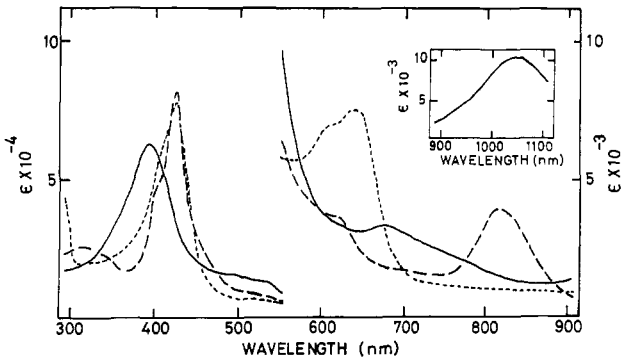


FIGURE 1: Optical absorption spectra of iron(III) 5-oxyoctaethylporphyrin dimer (**5**) (—) in $CHCl_3$, bis(tosylmethyl isocyanide)iron(II) 5-oxyoctaethylporphyrin radical (**6a**) (– –) in 45 mM $TosCH_2NC$ in $CHCl_3$, and bis(pyridine)iron(II) 5-oxyoctaethylporphyrin radical (**6b**) (- - -) in 0.62 M C_5H_5N in $CHCl_3$. The ϵ values of **5** are per monomer unit. The spectrum of **6a** was recorded at 30 min after the addition of $TosCH_2NC$.

1972).

Sunner et al. (1986) found that dimerization takes place in FAB and reported ratios of dimer to monomer of 0.01 to 0.33 in the FAB spectra of several compounds with molecular weights of 90 to 205. The highest ratio of 0.33 was observed with glycerol and was attributed in part to its smallness. The FAB spectrum of the much larger compound A showed a total relative abundance at *m/e* 1207 and 1206 that was 0.48 of the total relative abundance at *m/e* 605, 604, 603, and 602. In contrast, the total relative abundance shown by the same dimer peaks in the FAB spectrum of **6a**, a monomeric compound, was only 0.01 of that shown by the unliganded monomer peaks. Thus, elemental analyses and FAB spectra both favor identification of compound A as the dimer **5** rather than as the methanolic adduct of the monomer **2**. The proposed structure of compound A is consistent with its electronic spectrum in $CHCl_3$, which suggested a conjugated porphyrin ring, and with its infrared spectrum in KBr, which showed no carbonyl band. The ESR spectrum indicated a high-spin ferric compound (Weissbluth, 1974), and the variation in effective magnetic moment with temperature was consistent with the behavior of a dimeric high-spin ferric complex (Schugar et al., 1969).

The addition of pyridine, tosylmethyl isocyanide, or imidazole to **5** resulted in changes in optical spectra attributable to the dissociation of **5** into monomers and the formation of adducts of the added compounds with the monomer. ESR spectra of the product from the addition of imidazole showed no signal at 293 K and signals typical of a low-spin ferric porphyrin in which $g_1 \neq g_2 \neq g_3$ at 77 K (Mims & Peisach, 1976). The IR spectrum showed no band in the carbonyl region. These properties indicated that the product of dissociation by imidazole was the enolate tautomer of bis(imidazole)iron(III) 5-oxyoctaethylporphyrin (**2b**). The products of the addition of tosylmethyl isocyanide and pyridine differed in their properties from **2b** and from each other.

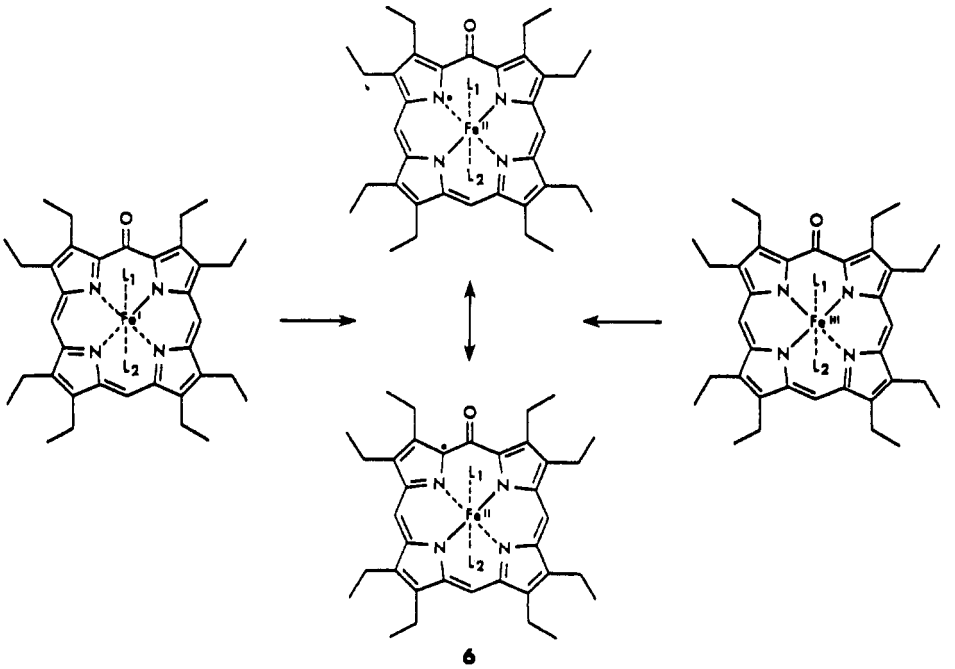
The electronic spectrum of compound B, which resulted from the reaction of **5** with $TosCH_2NC$, is shown in Figure 1. The fact that $TosCH_2NC$ is a strong ligand of ferrous porphyrins suggested that compound B is a ferrous porphyrin compound. The magnetic susceptibility of compound B showed one unpaired electron per iron atom, and its ESR spectrum showed that the electron was not localized at the iron atom but was in the oxyporphyrin ring. A strong signal at $g = 2.012$ at room temperature indicated that the dimer (**5**) had dissociated into two radical molecules. Therefore, the structure of compound B was determined to be bis(tosylmethyl isocyanide)iron(II) 5-oxyoctaethylporphyrin π-neutral radical (**6a**).

The optical spectrum of monomer C from the reaction of **5** with pyridine differed markedly from that of **6a**, the tosylmethyl isocyanide adduct (Figure 1). The presence of a carbonyl band in the IR spectrum of compound C indicated a structure different from that of **2b**, the imidazole adduct. The ESR spectrum of compound C at 295 K, like that of **2b**, showed no signal; however, the spectrum at 103 K, with $g_\perp = 2.37$ and $g_\parallel = 1.71$, differed from the typical low-spin ferric porphyrin spectrum shown by **2b**. Sano et al. (1981) reported that the ESR spectrum of oxymesohemin in pyridine/NaOH at 77 K was characteristic of high-spin iron(III) liganded with a hydroxy group. Addition of acid to pH 9.5 resulted in an unusual ESR spectrum with $g_\perp = 2.30$ and $g_\parallel = 1.76$. The iron(I) state was inferred by analogy with published data showing $g_\perp = 2.26$–2.32 and $g_\parallel = 1.93$–1.94 in iron(I) tetraphenylporphyrin (Cohen et al., 1972; Lexa et al., 1974; Kadish et al., 1975). Oxyprotoheme in aqueous pyridine showed nearly the same ESR spectrum ($g_\perp = 2.30$ and $G_\parallel = 1.78$) at 77 K and was likewise assigned the iron(I) state. Sodium was used in the chemical reduction of iron(III) tetraphenylporphyrin to iron(I) tetraphenylporphyrin (Cohen et al., 1972; Lexa et al., 1974). Formation of the postulated iron(I) state of oxyheme in the absence of external reductant requires the transfer of two electrons from the porphyrin ring to iron (Sano et al., 1981); however, iron(III) is not so strong an oxidant, nor is porphyrin so strong a reductant, to permit such a reaction. A two-electron oxidation of a porphyrin results in a π-dication or an isoporphyrin (Dolphin et al., 1970). In either structure the environment of the iron in oxyheme would be different from that of iron in iron(I) tetraphenylporphyrin. The similarity of the ESR spectra of oxyheme to that of iron(I) tetraphenylporphyrin may therefore be coincidental and not indicative of the oxidation state of the iron of oxyheme.

Bonnett and Dimsdale (1972) reported the preparation of aquairon(III) oxyoctaethylporphyrin (**2a**) by insertion of iron into the oxyporphyrin and described the conversion of this compound to the bis(pyridine) adduct (**2c**). This procedure did not yield **2a** in the present work but instead produced a mixture of the dimer **5** and octaethylbiliverdin. Addition of imidazole to **5** produced the enolate form of **2b**, a low-spin iron(III) oxyporphyrin. In contrast, addition of pyridine to **5** did not produce **2c** but resulted in compound C, a low-spin species with a carbonyl band in the IR and an unusual ESR spectrum not associated with low-spin iron(III) porphyrins (Mims & Peisach, 1976). Mössbauer and NMR spectra of the bis(pyridine) adducts of oxymesohemin and oxyprotohemin also were inconsistent with an iron(III) structure (Sano et al., 1981, 1986).

Identification of compound C as bis(pyridine)iron(II) 5-oxyoctaethylporphyrin π-neutral radical (**6b**) was supported by its optical spectrum and by spectral data on analogous compounds. Its optical spectrum resembled those of nickel(II) tetraphenylporphyrin π-cation radical perchlorate (Dolphin et al., 1975) and ruthenium(II) octaethylporphyrin π-cation radical bromide, $[Ru^{II}OEP(CO)]^{\bullet+}Br^-$ (Morishima et al., 1984). The latter compound, like **6b**, did not have a detectable radical signal in its ESR spectrum at room temperature. An apparent absence or diminution of a radical signal may be due to extreme broadening of the signal. Coupling between iron and a radical electron has been postulated as a possible basis for broadening of the radical signal in an ESR spectrum. (Schulz et al., 1979; Phillippi & Goff, 1982). Low-spin

Scheme I



6

a $L_1 = L_2 = TosCH_2NC$

b $L_1 = L_2 = C_5H_5N$

c $L_1 = C_5H_5N$, $L_2 = O_2$

d $L_1 = C_5H_5N$, $L_2 = CO$

e $L_1 = C_5H_5N$, $L_2 = TosCH_2NC$

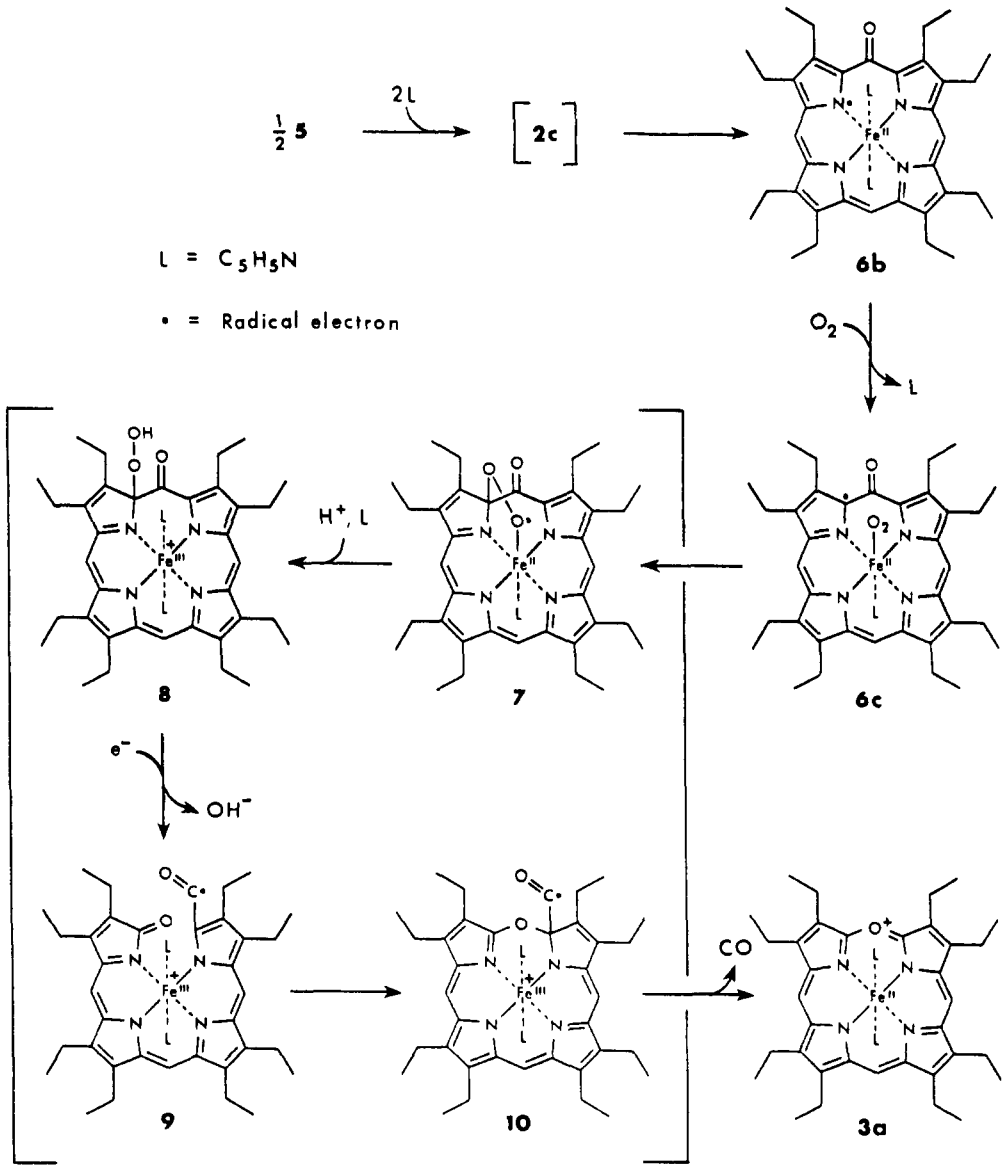
iron(II) ($S = 0$) in **6b** would be unable to couple with a radical electron; however, if partial transfer of spin density from porphyrin π radical to iron takes place so that $S \neq 0$, coupling may result in enhanced electron spin relaxation and broadened ESR spectra. Morishima et al. (1984) have proposed an alternative mechanism based on their NMR studies of $[Ru^{II}OEP(CO)]^{\bullet+}Br^-$ to account for the broadened ESR spectrum (Schulz et al., 1979) of horseradish peroxidase (HRP) compound I. The NMR spectrum of this ruthenium π-cation radical, which did not show a radical signal in its ESR spectrum, was interpreted as being due to the mixing of the $^2A_{1u}$ and $^2A_{2u}$ states. The possibility of enhanced electron spin relaxation caused by fast exchange between $^2A_{1u}$ and $^2A_{2u}$ porphyrin radical states was suggested to account for the NMR and ESR spectra of the porphyrin π-cation of HRP compound I.

In light of the possible mechanisms that have been advanced to account for broadening of the radical signal in the ESR spectrum of a porphyrin π radical, failure to detect a well-defined radical signal seems not necessarily to preclude a π-radical structure. Other results with structural analogues of **6b** are in accord with an iron(II) porphyrin π-neutral radical structure for this pyridine complex. Mössbauer spectra of both bis(pyridine)oxymesohemin and bis(pyridine)oxyprotohemin were consistent with ferrous low-spin structures, and the NMR spectrum of bis(pyridine)oxyprotohemin showed extensive spin delocalization in the porphyrin π system (Sano et al., 1981, 1986). Sano et al. (1986) suggested that bis(pyridine)oxyprotohemin is best described as iron(II) oxyprotoporphyrin π-neutral radical mixed with an iron(I) species with the radical species increasing with increasing temperature. Scheme I shows the iron(I) isoporphyrin, iron(II) porphyrin π radical, and iron(III) porphyrin representations of oxyoctaethylhemin. Available data as discussed above favor the iron(II) radical structure for **6b** as well as for **6a**.

The ESR and optical spectra of a pair of ruthenium π-cation radicals, $[Ru^{II}OEP(CO)]^{\bullet+}ClO_4^-$ and $[Ru^{II}OEP(CO)]^{\bullet+}Br^-$, are different (Morishima et al., 1984). The radical signal of the perchlorate, but not of the bromide, was seen by ESR at room temperature. The optical spectra of the bromide and perchlorate resembled those assigned (Dolphin et al., 1974) to the $^2A_{1u}$ state and the $^2A_{2u}$ state, respectively, of metalloporphyrin π-cation radicals. However, NMR data on the perchlorate were interpreted as showing that it is predominantly in the $^2A_{1u}$ state. Temperature dependences of the NMR and optical spectra of the bromide were explained by the assumption that this radical is in a thermal equilibrium between the $^2A_{1u}$ and $^2A_{2u}$ electronic states. It is unlikely that **6a** and **6b** are in the same electronic state in view of the difference between their optical spectra (Figure 1), which resemble the difference between the spectra of ligand-dependent ground states of cobalt(III) octaethylporphyrin radicals (Dolphin et al., 1973). By analogy with the ground state assignments of the cobalt compounds by their optical spectra, **6a** is in the $^2A_{1u}$ state and **6b** is in the $^2A_{2u}$ state. On the other hand, by analogy with $[Ru^{II}OEP(CO)]^{\bullet+}Br^-$, which, like **6b** showed no radical signal, **6b** may be a mixture of the two states. Radical signals that appeared when **6b** was exposed to O_2, CO, or $TosCH_2NC$ indicated that displacement of a pyridine ligand by a strong-field axial ligand of iron(II) porphyrins resulted in an electronic structure like that of **6a** for **6c**, **6d**, and **6e**.

Although the reaction of **2** or **6** with O_2 in pyridine to produce **3** and CO takes place in the absence of added reducing

Scheme II



agent (Bonnett & Dimsdale, 1972), the stoichiometry of this reaction requires the addition of a reducing equivalent or expulsion of an oxidizing equivalent, as discussed by Lagarias (1982). Neither of the two schemes proposed by Sano et al. (1986) accounted for this stoichiometry. The release of $^1/_2H_2O_2$, presumably as hydroxyl radical, was postulated by Fuhrhop et al. (1975a); therefore, a spin-trapping experiment with DMPO was conducted. A signal pattern similar to that of the DMPO adduct of superoxide (Harbour & Bolton, 1975) was detected and was assigned to the DMPO adduct of **7**, but signals assignable to the DMPO–hydroxyl radical adduct were not detected.

Our proposed mechanism for the conversion of iron(III) oxyoctaethylporphyrin to iron(II) octaethylverdohemochrome by oxygen in the presence of pyridine is shown in Scheme II. In the present work, unliganded iron(III) octaethylporphyrin was found, not as a monomer, but as the dimer **5**. Pyridine dissociates the dimer to bis(pyridine)iron(II) 5-oxyoctaethylporphyrin radical (**6b**), and exposure to oxygen results in the displacement of a pyridine ligand of **6b** by an oxygen molecule to produce the radical **6c**. The oxygen ligand is transferred to the C-1 carbon adjacent to the carbonyl carbon via **7** to form the peroxy adduct **8**. The yield, 47%, of **3a** from **5** by the action of O_2 was increased to 80% with the addition of ascorbate or phenylhydrazine. A one-electron reduction of **8** is postulated to expel an oxygen atom as hydroxide and open the porphyrin ring to form **9**. In the absence of added reductant, a component of the reaction mixture must be the source of the electron. Abstraction of an electron from **6b** by the peroxy adduct **8** may oxidize the former to an iron(IV) porphyrin or an iron(III) porphyrin π-cation radical (Phillippi & Goff, 1982). The open tetrapyrrole structure of **9** is held in a cyclic configuration by the central atom, as in cyclic metal complexes of biliverdin (Fuhrhop et al., 1975b; Bonfiglio et al., 1983), until the oxaporphyrin ring is formed to produce **10**. The structural relationship between **9** and **10** is analogous to that between open-ringed iron biliverdin and its closed-ring hemiketal isomer (Saito & Itano, 1982). Octaethylverdohemochrome (**3a**) results from the release of CO from **10**.

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Registry No. **1a**, 28755-93-3; **1b**, 19496-63-0; **2b**, 108104-01-4; **2c**, 108119-89-7; **3a**, 108104-02-5; **3b**·OH⁻, 108104-03-6; **3c**·OH⁻, 108104-04-7; **4**, 108104-05-8; **4** (pyridine derivative), 108104-06-9;

5, 108104-07-0; **6a**, 108148-03-4; **6c**, 108148-04-5; **6d**, 108119-90-0; **6e**, 108119-91-1; **7**, 108119-92-2; $FeCl_3$, 7705-08-0; $TosCH_2NC$, 36635-61-7; oxyoctaethylporphyrin, 108104-08-1; benzoyl chloride, 98-88-4; 5-benzoyloxyoctaethylporphyrin, 19512-49-3; imidazole, 288-32-4; phenylhydrazine hydrochloride, 59-88-1; ascorbic acid, 50-81-7.

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