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Biochemistry

β-*meso*-Phenylbiliverdin IXα and *N*-phenylprotoporphyrin IX, products of the reaction of phenylhydrazine with oxyhemoproteins

(*meso*-tolylbiliverdin/*N*-tolylprotoporphyrin/Heinz body anemia/oxyhemoglobin/oxymyoglobin)

SETSUO SAITO AND HARVEY A. ITANO

Department of Pathology, D-006, University of California, San Diego, La Jolla, California 92093

Contributed by Harvey A. Itano, June 12, 1981

ABSTRACT **Oxyhemoglobin and oxymyoglobin were allowed to react aerobically with phenylhydrazine and *p*-tolylhydrazine. The chloroform extract of each reaction mixture, after treatment with H_2SO_4/methanol, yielded a blue pigment and a green pigment, which were identified by electronic absorption, mass, and proton NMR spectroscopy as the dimethyl esters of β-*meso*-arylbiliverdin IXα and *N*-arylprotoporphyrin IX, respectively. *N*-Phenylprotoporphyrin IX dimethyl ester formed complexes with Zn^{2+}, Cd^{2+}, and Hg^{2+} but not with other cations. The proton NMR spectrum of the zinc complex suggested binding of the phenyl group to one of the two pyrrole rings of protoporphyrin IX with a propionic acid substitutent. The effectiveness of phenylhydrazine as an inducer of Heinz body formation may be due to destabilization of the hemoglobin molecule by the replacement of heme with phenyl adducts of biliverdin and protoporphyrin.**

The green hemoglobin produced in the reaction of hemoglobin with phenylhydrazine was found by Kiese and Seipelt (1) to differ from verdoglobins produced by other reagents. Lemberg and Legge (2) showed that biliverdin production was increased in the erythrocytes of rabbits treated with phenylhydrazine, and they concluded that this *in vivo* result of phenylhydrazine activity corresponded to the *in vitro* formation of biliverdin in the coupled oxidation of hemoglobin and ascorbic acid. Beaven and White (3), on the other hand, did not obtain biliverdin in the *in vitro* reaction of phenylhydrazine with oxyhemoglobin; instead they isolated a green pigment with an absorption band in the Soret region suggestive of an intact porphyrin ring.

Studies from this laboratory indicated that substituents on the benzene ring affected the hemolytic activity of substituted phenylhydrazine and the binding of substituted phenyldiazenes to methemoglobin in parallel fashion (4). Subsequent studies showed, however, that the hemolytic activity of ring-substituted nitrosobenzenes was not related to their affinities as ligands of deoxyhemoglobin (5).

A two-electron transfer to oxyhemoglobin was suggested as the initial step in the degradation of heme mediated by ascorbic acid (6), and the same transfer was postulated in the reaction of phenylhydrazine with oxyhemoglobin (4). Although the processes may be initiated in the same way, different reported products, biliverdin with ascorbic acid (2) and an intact porphyrin with phenylhydrazine (3), indicate that subsequent steps are different. We therefore undertook to isolate and characterize the products of heme degradation when hemoglobin is treated with arylhydrazines, and we found two types of compounds: one is a biliverdin IXα derivative with an aryl group on a *meso* carbon, and the other is a protoporphyrin IX derivative with an aryl group on a pyrrole nitrogen.

EXPERIMENTAL PROCEDURES

Materials and Methods. To a solution of metmyoglobin (5 g, type III from horse heart, Sigma) in potassium phosphate buffer (pH 7.4, 0.01 M, 200 ml), a 50-fold excess of powdered $Na_2S_2O_4$ was added under N_2. This mixture was applied onto a column of Sephadex G-25 (5 cm inside diameter × 30 cm) equilibrated with phosphate buffer (pH 7.4, 0.01 M) and was eluted from the column with the same buffer. The eluate was dialyzed against phosphate buffer (pH 7.4, 0.1 M, 4 liters) to obtain a solution of oxymyoglobin (3.58 g, 72% yield). Washed human erythrocytes were lysed with 3 vol of distilled water and centrifuged at 12,000 × *g* for 90 min to obtain a clear solution of hemolysate. This solution was dialyzed against phosphate buffer (pH 7.4, 0.1 M, 4 liters), and the resulting dialysate was used for reactions of oxyhemoglobin. Biliverdin IX, obtained by coupled oxidation of hemin, myoglobin, or hemoglobin with ascorbic acid (7, 8), was esterified in 7% H_2SO_4 in methanol.* The isomers of biliverdin IX dimethyl ester were separated by thin-layer chromatography (TLC) (9). Phenylhydrazine·HCl and *p*-tolylhydrazine HCl (Aldrich) were recrystallized from ethanol before use. SilicAR cc-7 Special (Mallinckrodt) was used for column chromatography. Uniplate (Silica Gel G, Analtech) was used for analytical and preparative TLC. Electron impact mass spectra were obtained on an LKB type 9000 spectrometer at an ionizing energy of 70 eV by the direct inlet method. Field desorption mass spectra were obtained on a Kratos/AEI MS-902 instrument. Optical spectra were recorded on a Cary model 17 spectrophotometer. Proton nuclear magnetic resonance (NMR) spectra of samples in C^2HCl_3 solution containing internal tetramethylsilane were recorded with a custom-designed 360-MHz spectrometer with 200–400 pulses in the Fourier-transform mode. High-performance liquid chromatography was carried out with the Altex model 334 system. A Whatman Partisil 10-PAC column (4.6 × 250 mm) preceded by a short Partisil 10 protective precolumn was used. Mass spectra of the four isomers of biliverdin IX dimethyl ester and the NMR spectrum of biliverdin IXα dimethyl ester were obtained for use as standards.

Blue (I) and Green (II) Pigments from the Reaction of Phenylhydrazine with Oxymyoglobin. A solution of phenylhydrazine·HCl (188 mg) in phosphate buffer (pH 7.4, 0.1 M, 100 ml) was added to 500 ml of 260 μM oxymyoglobin in the same buffer, and the reaction mixture was allowed to stand for 1 hr under aerobic conditions with occasional shaking. Acetic acid (400 ml) and concentrated HCl (100 ml) were then added to 0°C. After 20 hr at 4°C, the reaction mixture was extracted twice with chloroform (300 ml and 150 ml). The extracts were gathered, washed

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Abbreviations: TLC, thin-layer chromatography; Fe^{II}, deoxyhemoprotein; $Fe^{II}O_2$, oxyhemoprotein.

* All reagent percentages and solvent ratios are vol/vol.

with distilled water, dried over anhydrous Na_2SO_4, filtered, and evaporated to obtain residue A (99.5 mg, 59.9% yield from phenylprotoporphyrin, M_r 638). A solution of this residue in 7% H_2SO_4 in methanol (100 ml) at 0°C was allowed to stand for 18 hr at 4°C, diluted with cold distilled water (150 ml), and extracted twice with chloroform (200 ml and 100 ml). The extracts were treated as described above to obtain a residue that showed a minor blue spot (R_F: 0.47 in 4:1 benzene/acetone, 0.90 in 22:3 chloroform/ethanol), a major green spot (R_F: 0.02 in 4:1 benzene/acetone, 0.20 in 22:3 chloroform/ethanol), and some brown spots by TLC. The residue was applied onto a silica gel column (1.5 cm inside diameter × 12 cm) and eluted in succession with the following solvents: 100% chloroform (100 ml), 3% acetone in chloroform (50 ml), 5% acetone in chloroform (50 ml), 5% methanol in chloroform (150 ml), and 10% methanol in chloroform (300 ml). The 5% acetone eluate was evaporated to obtain a blue oil (I, 13 mg, 12% yield from residue A), which was further purified by preparative TLC (silica gel, 4:1 benzene/acetone) for spectral determinations. Mass spectrum *m/e* (relative intensity): 686 (parent ion M^+, 2.0), 567 (14.0), 478 (3.6), 404 (3.6), 402 (3.4), 390 (56.8), 376 (100), 361 (4.5), 359 (3.6), 347 (4.0), 345 (5.9), 317 (20.5), 314 (9.8), 303 (20.0), 300 (36.0), 287 (50.0), 241 (22.7), 227 (40.9), 199 (27.3), 181 (22.6), 180 (22.7), 122 (22.7), 155 (13.6), 108 (40.9). NMR spectrum (in C^2HCl_3; δ in ppm; m, multiplet; s, singlet) δ: 1.60 (CH_3), 1.94 (CH_3), 2.01 (CH_3), 2.03 (CH_3), 2.54 (4H, m, Ar—CH_2 × 2), 2.88 (4H, m, —CH_2CO × 2), 3.68 (6H, OCH_3 × 2), 5.77 (4H, m, —CH=CH_2 × 2), 5.95 (1H, s, H-15), 6.60–6.70 (2H, m, —C*H*=CH_2 × 2), 6.67 (1H, s, H-10), 7.26 (5H, —C_6H_5), 8.50 (3H, broad s, NH × 3). Electronic absorption spectrum $\lambda_{max}^{CHCl_3}$ *nm* (ε_{mM}): 373 (50.1), 631 (14.5). The 10% methanol eluate was evaporated, and the resulting residue was purified by preparative TLC (silica gel, 85:15 chloroform/methanol) to obtain a green pigment (II, 42.3 mg, 40.7% yield from residue A). Field desorption mass spectrum *m/e*: 666 (M^+), 667 (M^+ + 1). NMR spectrum (in C^2HCl_3) δ: 2.26 (2H *ortho* protons of phenyl group), 2.80–3.30 (4H, —CH_2CO × 2), 3.30–3.65 (18H, CH_3 × 4 and OCH_3 × 2), 4.20–4.50 (4H, Ar—CH_2— × 2), 4.75 (2H, *meta* protons of phenyl group), 5.47 (1H, *para* proton of phenyl group), 6.10–6.65 (4H, —CH=CH_2 × 2), 7.80–8.25 (2H, —C*H*=CH_2 × 2), 10.10–10.50 (4H, *meso* protons). Electronic absorption spectrum $\lambda_{max}^{CHCl_3}$ nm (ε_{mM}): free base 426 (140.6), 517 (14.0), 550 (9.1), 612 (6.0), 670 (2.6); univalent cation 412 (166.3), 555 (10.3), 574 (15.8), 595 (11.7), 615 (8.0); Zn^{2+} complex 442 (158.3), 548 (12.2), 602 (14.2), 645 (4.4); Cd^{2+} complex 438 (148.3), 565 (12.0), 607 (13.3), 653 (3.8); Hg^{2+} complex 450 (143.3), 554 (12.4), 609 (14.0), 660 (4.7).

Blue (III) and Green (IV) Pigments from the Reaction of *p*-Tolylhydrazine with Oxymyoglobin. To a solution of oxymyoglobin (150 μM) in phosphate buffer (pH 7.4, 0.1 M, 300 ml), *p*-tolylhydrazine·HCl (117 mg) was added. The procedures described above for the phenylhydrazine reaction were followed to obtain residue B (54.7 mg, 55.9% yield from tolylprotoporphyrin, M_r 652), a blue pigment (III, 6 mg, 10.2% yield from residue B), and a green pigment (IV, 32.8 mg, 57.4% yield from residue B). Blue pigment III mass spectrum *m/e* (relative intensity): 700 (M^+, 3.1), 579 (6.5), 505 (6.0), 404 (40.0), 390 (100), 314 (7.0), 300 (20.0), 287 (4.0), 241 (3.1), 227 (6.0), 225 (4.2), 213 (11.3), 181 (4.0). Green pigment IV field desorption mass spectrum *m/e*: 680 (M^+) and 681 (M^+ + 1). Electronic absorption spectrum $\lambda_{max}^{CHCl_3}$ nm (ε_{mM}): free base 426 (127.3), 518 (13.3), 550 (9.0), 612 (6.8), 670 (2.8); univalent cation 420 (159.4), 555 (10.8), 573 (14.8), 595 (10.0), 523 (8.4); Zn^{2+} complex 432 (141.0), 547 (9.9), 602 (14.0), 643 (4.5).

Blue (I) and Green (II) Pigments from the Reaction of Phenylhydrazine with Oxyhemoglobin. To a solution of oxyhemoglobin (227 μM) in phosphate buffer (pH 7.4, 0.1 M, 600 ml), a solution of phenylhydrazine·HCl (800 mg) in the same buffer (100 ml) was added. The procedure described above to obtain residue A was used to obtain residue C (405 mg, 57.3% yield from phenylprotoporphyrin, M_r 638). The crude product from the treatment of residue C with 7% H_2SO_4 in methanol was applied onto a silica gel column (5 cm inside diameter × 20 cm) and eluted with the following solvents: 100% chloroform (200 ml), 5% acetone in chloroform (200 ml), 5% methanol in chloroform (200 ml), and 10% methanol in chloroform (400 ml). The 5% acetone eluate was evaporated to give a residue that showed a major blue spot (R_F: 0.47 in 4:1 benzene/acetone and a minor blue spot (R_F: 0.53 in 4:1 benzene/acetone) that overlapped a brown spot by TLC. The major blue pigment (43 mg, 9.9% from residue C) was isolated and purified by preparative TLC. This blue pigment was the same as pigment I obtained from the reaction of oxymyoglobin with phenylhydrazine according to its TLC behavior, electronic absorption spectrum, and NMR spectrum. The 10% methanol eluate was evaporated to give a residue from which a green pigment (184 mg, 43.5% yield from residue C) was isolated by preparative TLC. The TLC and spectral properties of this green pigment were the same as those of pigment II from the reaction of oxymyoglobin with phenylhydrazine.

Zinc Complex of Green Pigment II. Zinc acetate (500 mg) in methanol (20 ml) was added at room temperature to a chloroform solution (100 ml) of green pigment II (50 mg), and the solvents were removed by evaporation. The residue was extracted with chloroform, and the extract was evaporated to obtain a residue that showed a major green spot (R_F: 0.39) accompanied by two small green spots (R_F: 0.33 and 0.31) on TLC (85:15 chloroform/methanol). The major component (33 mg, 60% yield) was isolated by preparative TLC and purified by silica gel column chromatography. The zinc complex of green pigment II eluted as a single peak by high-performance liquid chromatography (4:1:2 *n*-hexane/tetrahydrofuran/methanol). NMR spectrum (C^2HCl_3; *J* coupling constant; dd, double doublet) δ: 2.00 (2H dd, J_1 = 14 Hz, J_2 = 5 Hz, *ortho* protons of phenyl group), 2.82 and 3.08 (4H —CH_2CO— × 2), 3.42–3.63 (18H, OCH_3 × 2 and aryl—CH_3 × 4), 4.10–4.40 (4H, aryl—CH_2— × 2), 5.01 (2H, dd, J_1 = 14 Hz, J_2 = 5 Hz, *meta* protons of phenyl group), 5.57 (1H, dd, J_1 = 14 Hz, J_2 = 5 Hz, *para* proton of phenyl group), 6.08–6.45 (4H, m, —CH=CH_2 × 2), 7.98–8.30 (2H, m, —C*H*=CH_2 × 2), 10.05–10.25 (4H, m, *meso* protons).

RESULTS

Structure of Blue Pigments I and II. Blue pigment I from the reations of both oxymyoglobin and oxyhemoglobin with phenylhydrazine and blue pigment III from the reaction of oxymyoglobin with *p*-tolylhydrazine gave electronic absorption spectra similar to the spectrum of biliverdin IX dimethyl ester (7, 10). The mass spectra of I and III showed molecular ion peaks at *m/e* 686 and 700, respectively. These molecular ions suggested that a phenyl group (mass unit 77) and a tolyl group (mass unit 91) replaced a proton on biliverdin IX dimethyl ester (M_r = 610) in pigment I and pigment III, respectively. The mass spectra of biliverdin IXα dimethyl ester and biliverdin IXγ dimethyl ester show a characteristic fragment ion at *m/e* 300 (refs. 7 and 10 and present work), consistent with structure Va of Fig. 1. On the other hand, the mass spectra of biliverdin IXβ dimethyl ester and biliverdin IXδ dimethyl ester show a significant ion at *m/e* 360 (ref. 7 and present work), consistent with structure VI. The mass spectra of pigments I and III showed characteristic fragment ions as base peaks at *m/e* 376 and *m/e*

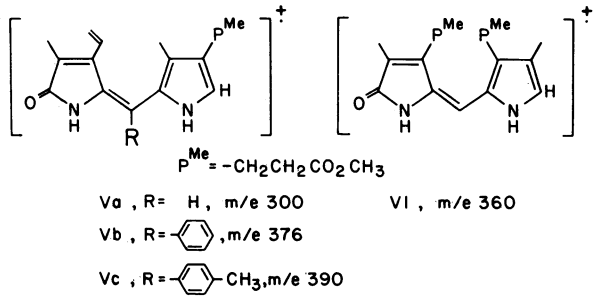

FIG. 1. Characteristic fragment ions of biliverdin IX dimethyl esters and blue pigments I and III.

390, consistent with structures **Vb** and **Vc**, in which a phenyl group and a tolyl group, respectively, are attached to ion **Va**. These data indicate that pigments **I** and **III** are derivatives of biliverdin dimethyl esters of type IXα or IXγ, not of type IVβ or IXδ.

The NMR spectrum of blue pigment **I**, shown in Fig. 2, differs significantly from that of biliverdin IXγ dimethyl ester (11, 12). While the spectrum is similar to that of biliverdin IXα, differences in signal patterns are discernible in the regions of the protons on the *meso* carbons and pyrrole methyl groups. Differences between these NMR spectra are summarized in Table 1. The spectrum of biliverdin IXα dimethyl ester shows

Table 1. Comparison of chemical shifts from proton NMR spectra at 360 MHz

Protons*	δ, ppm (no. of hydrogens)	
	Biliverdin IXα dimethyl ester	Blue pigment I
Aryl methyl	1.85 (3H), 2.06 (3H), 2.11 (3H), 2.16 (3H)	1.60 (3H), 1.94 (3H), 2.01 (3H), 2.03 (3H)
Ester methyl	3.68 (6H)	3.68 (6H)
meso H-5, H-15	5.96 (1H), 6.00 (1H)	5.95 (1H)
meso H-10	6.76 (1H)	6.67 (1H)
Vinyl —CH=CH_2	5.38 and 5.61 (2H) 5.98 and 6.06 (2H)	5.48–5.67 (4H)
Vinyl —CH=CH$_2$	6.50 (1H), 6.62 (1H)	6.36–6.50 (2H)

* See Figs. 2 and 3 for structures and proton designations.

three *meso* protons; the spectrum of pigment **I** shows only two *meso* protons, of which one is the γ proton and the other is either the β or the δ proton. In biliverdin IXα, the δ position is between two methyls, while the β position is between a methyl and a vinyl. In the spectrum of pigment **I**, the signals of one of the four methyls and one of the two vinyls are shifted upfield. These shifts indicate that the phenyl group is on the β-*meso* carbon. Accordingly, pigment **I** is β-*meso*-phenylbiliverdin IXα methyl ester, and pigment **III** is β-*meso*-*p*-tolylbiliverdin IXα dimethyl ester (Fig. 3).

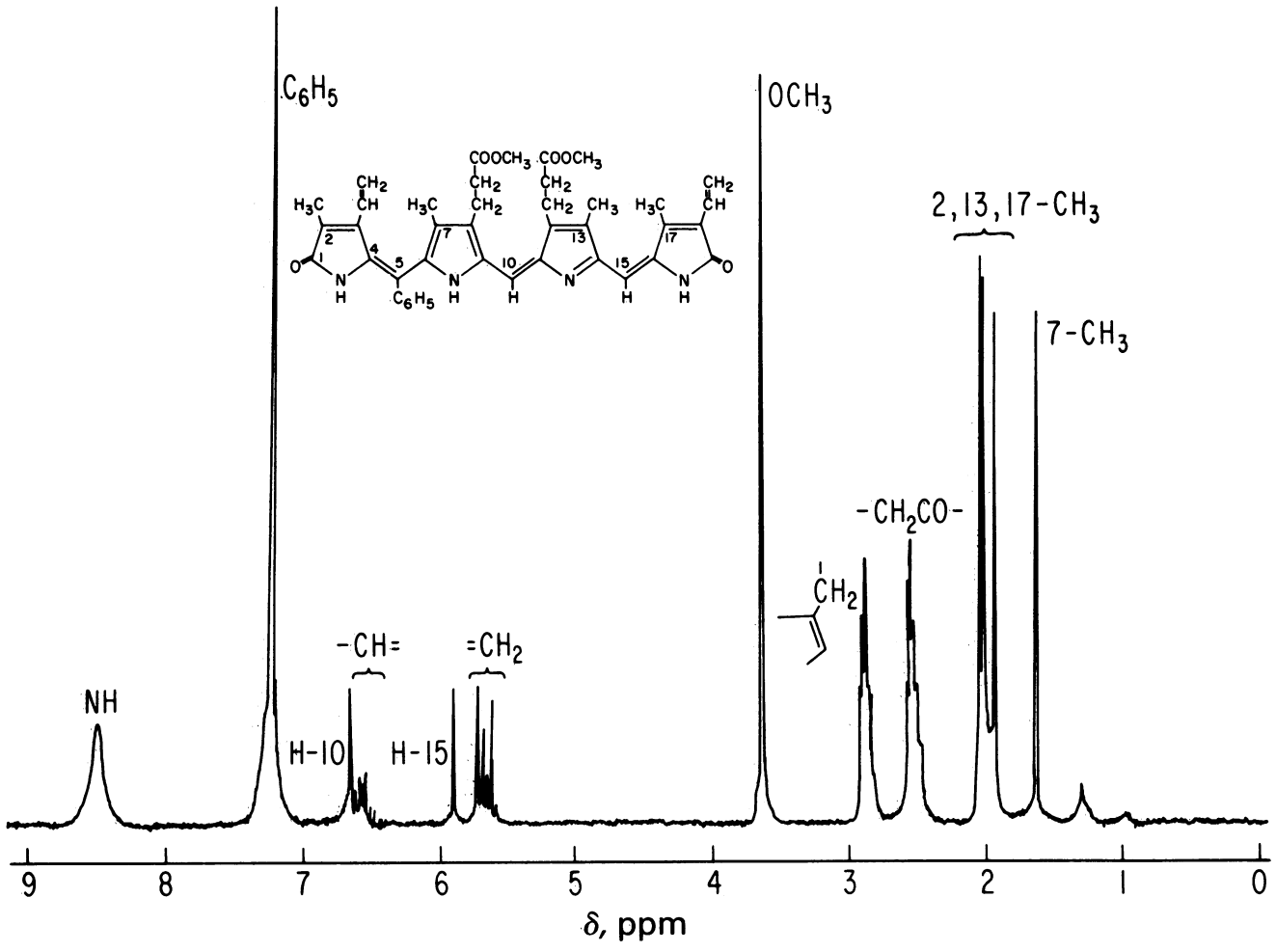

FIG. 2. Proton NMR spectrum at 360 MHz of β-*meso*-phenylbiliverdin IXα dimethyl ester in C^2HCl_3.

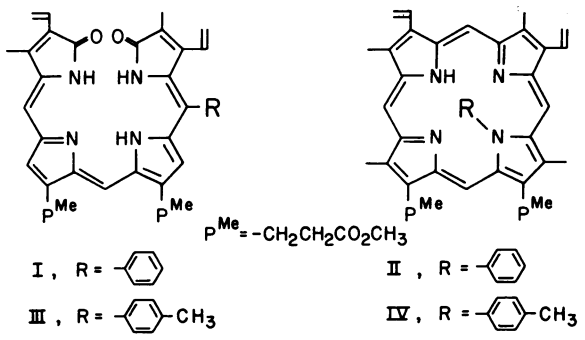

FIG. 3. Structures of β-*meso*-arylbiliverdins IXα dimethyl ester (blue pigments I and III) and *N*-arylprotoporphyrin IX dimethyl ester (green pigments II and IV).

Structure of Green Pigments II and IV. Green pigment IIwas obtained from the reactions of both oxymyoglobin and oxyhemoglobin with phenylhydrazine. Its field desorption mass spectrum showed peaks at *m/e* 666 (M^+) and 667 ($M^+ + 1$). The field desorption mass spectrum of green pigment IV from the reaction of oxymyoglobin with *p*-tolylhydrazine showed peaks at *m/e* 680 (M^+) and 681 ($M^+ + 1$). These results are consistent with the substitution of one phenyl group (mass unit 77) and one tolyl group (mass unit 91), respectively, for a proton in protoporphyrin IX dimethyl ester (M_r 590). All of the carbon protons of protoporphyrin IX dimethyl ester (13) were assignable on the NMR spectrum of the zinc complex (Fig. 4). The protons of the *ortho, meta,* and *para* positions of the phenyl group, which are confirmed by the spin decoupling method, were shifted to higher fields at chemical shifts of δ = 2.00, 5.01, and 5.57, respectively.

The signal patterns of the vinyl group at δ 6.09–6.45 and 7.98–8.30 are similar to those of protoporphyrin IX dimethyl ester (13); on the other hand, the signals of the methylene protons of the propionic acid methyl ester group are different. The signals of the methylene protons adjacent to the pyrrole ring (δ 4.10–4.40) are broadened, and the signals of the methylene protons adjacent to the ester group are separated at chemical shifts of 2.82 and 3.08. These results suggest that the phenyl group is attached to one of the two pyrrole rings with a propionic acid methyl ester substituent (14, 15).

Metal ions (Mg^{2+}, V^{4+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Sn^{2+}, Sb^{2+}, Al^{3+}) were added to chloroform solutions of II; only Zn^{2+}, Cd^{2+}, and Hg^{2+} formed complexes. The electronic absorption spectra of the free base, the univalent cation, and the Zn^{2+} complex of green pigment II are shown in Fig. 5. These spectra are like those of other *N*-substituted porphyrin derivatives (14, 16, 17). Similar spectra were obtained with green pigment IV. Green pigments II and IV are therefore *N*-phenylprotoporphyrin IX dimethyl ester and *Np*-tolylprotoporphyrin IX dimethyl ester, respectively (Fig. 3).

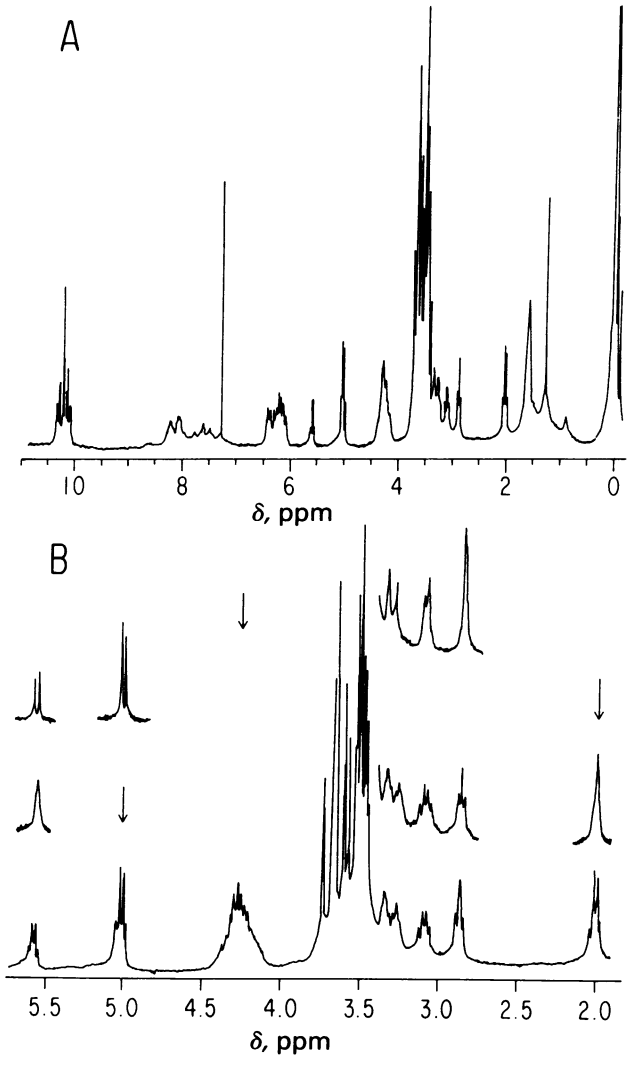

FIG. 4. (*A*) Proton NMR spectrum at 360 MHz of zinc *N*-phenylprotoporphyrin IX dimethyl ester in C^2HCl_3. (*B*) Decoupling of phenyl protons and sidechain methylene protons.

DISCUSSION

The formation of β-*meso*-phenylbiliverdin IXα requires two site-specific processes, phenylation of the β-*meso* carbon and oxidative cleavage of the α-*meso* position of protoporphyrin IX. Specific arylation of the β-*meso* carbon is a novel reaction, but specific α cleavage occurs both in the physiological degradation of heme and in the coupled oxidation of myoglobin and ascorbate (8). A two-electron reduction of oxyhemoglobin ($Fe^{II}O_2$) was postulated as the initial reaction in coupled oxidation (18). The transient product of this reaction, formulated as $Fe^{II}H_2O_2$, is the immediate precursor of *meso*-hydroxyheme, the first chemically identifiable intermediate in the oxidative cleavage of heme (6). This postulated mechanism was confirmed by the demonstration of *meso* oxidation when pyridine octaethylhem-

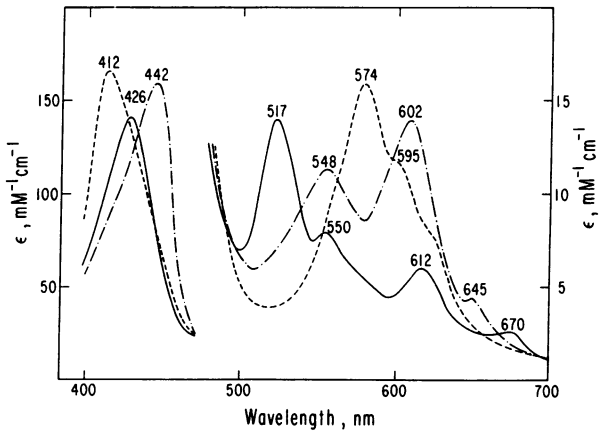

FIG. 5. Electronic absorption spectra of *N*-phenylprotoporphyrin IX dimethyl ester: ——, free base; ----, univalent cation; –·–, zinc complex.

ochrome was allowed to react with H_2O_2 (19). $Fe^{II}H_2O_2$ has also been represented as its anhydro derivative, $Fe^{IV}O$ (20).

The presence of *meso*-phenylbiliverdin coupled with the absence of unsubstituted biliverdin among the products of the reaction between phenylhydrazine and myoglobin indicates a close linkage between oxidative cleavage and *meso* phenylation in this reaction. A bimolecular reaction between oxyhemoglobin and phenylhydrazine (4) would simultaneously initiate both ring cleavage and ring phenylation.

$$Fe^{II}O_2 + C_6H_5NHNH_2 \rightarrow [Fe^{II}H_2O_2] + C_6H_5N{:}NH \quad [1]$$

$Fe^{II}H_2O_2$ is the postulated intermediate that leads to *meso* oxidation in coupled oxidation (6, 19). Phenyldiazene ($C_6H_5N{:}NH$) reacts rapidly with oxygen to become phenyldiazenyl radical (21), which immediately decomposes to phenyl radical and N_2 (22).

$$C_6H_5N{:}NH + O_2 \rightarrow C_6H_5N{:}N\cdot + H^+ + O_2^- \quad [2]$$

$$C_6H_5N{:}N\cdot \rightarrow C_6H_5\cdot + N_2 \quad [3]$$

Eqs. 1 and 2 account for the effect on O_2 tension of adding phenylhydrazine to a solution of oxyhemoglobin. A sharp drop in O_2 concentration followed by a spontaneous restoration of nearly three-fourths of the drop was recorded (23). These effects are attributable to the reduction of O_2 to superoxide radical (O_2^-) by phenyldiazene followed by the recovery of O_2 from O_2^-. Oxidation *in situ* of phenyldiazene from Eq. 1 by O_2 entering the heme crevice would provide a phenyl radical in the vicinity of the protoporphyrin group undergoing *meso* oxidation.

The formation of *N*-phenylprotoporphyrin requires that the phenyl group be attached to a pyrrole nitrogen before the process of oxidative cleavage becomes irreversible. A mechanism for its formation must at the same time account for its relatively high yield with respect to that of *β-meso*-phenylbiliverdin. The reaction of unmodified oxyhemoprotein with phenyldiazene or phenyl radical from the autooxidation of phenylhydrazine is unlikely to result in such a yield because autooxidation is only about 0.2% as fast as the reaction of phenylhydrazine with oxyhemoglobin (23). Phenyl radical from Eqs. 1–3 may add to a pyrrole nitrogen instead of to the *β-meso* carbon and result in displacement of the iron atom of heme, which is essential for *meso* cleavage (19). In the presence of an excess of phenylhydrazine, a second molecule of this compound may react with $Fe^{II}H_2O_2$ (or $Fe^{IV}O$) to restore Fe^{II}, aborting ring cleavage and at the same time producing another molecule of phenyldiazene.

$$Fe^{II}H_2O_2 + C_6H_5NHNH_2 \rightarrow Fe^{II} + 2H_2O + C_6H_5N{:}NH$$

$$\text{or } Fe^{IV}O + C_6H_5NHNH_2 \rightarrow Fe^{II} + H_2O + C_6H_5N{:}NH. \quad [4]$$

Formation of a second molecule of phenyldiazene agrees with the finding (23) that two molecules of O_2 per oxyheme were rapidly consumed in the reaction of an excess of phenylhydrazine with oxyhemoglobin.

Stability of the hemoglobin molecule depends partly on contacts between its heme groups and the side chains of the amino acid residues that surround them (24). When an inherited amino acid replacement alters these contacts, the molecule becomes unstable and precipitates as Heinz bodies, the formation of which is associated with hemolytic anemia (25). Some small compounds induce Heinz body hemolytic anemia even with normal hemoglobin; of these, phenylhydrazine is the most effective. Disruption of normal stabilizing contacts between heme and globin when heme is replaced with *meso*-phenylbiliverdin, *N*-phenylprotoporphyrin, or both, may be the physical basis for the effectiveness of phenylhydrazine.

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