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Akira Nakanishi*

Keiko Kinuta[†]

Tadashi Abe[‡]

Kenta Araki**

Yumi Yoshida^{††}

Shuang Liang^{‡‡}

Shun-Ai Li[§]

Kohji Takei[¶]

Masahiro Kinuta^{||}

*Okayama University,

[†]Okayama University,

[‡]Okayama University,

**Okayama University,

^{††}Okayama University,

^{‡‡}Okayama University,

[§]Okayama University,

[¶]Okayama University,

^{||}Okayama University,

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Akira Nakanishi, Keiko Kinuta, Tadashi Abe, Kenta Araki, Yumi Yoshida, Shuang Liang, Shun-Ai Li, Kohji Takei, and Masahiro Kinuta

Abstract

Administration of phenylhydrazine to rabbits resulted in the denaturation of hemoglobins in erythrocytes, causing the formation of intracellular precipitates known as Heinz bodies, severe hemolytic anemia, and reticulocytosis. To elucidate the molecular mechanism of the destabilization, we allowed human oxyhemoglobins to react aerobically with phenylhydrazine. After treatment with acetic acid/HCl and H₂SO₄/methanol, the chloroform extract contained blue-green pigments of major products accompanied by different minor products. Each product was isolated by column chromatography. By fast-atom-bombardment mass spectrometry (FAB-MS) and proton nuclear magnetic resonance (¹H-NMR) spectrometry, dimethyl esters of N-phenylprotoporphyrin IX and meso, N-diphenylprotoporphyrin IX were determined. Other major products also were determined to be dimethyl esters of triphenyl- and tetraphenyl-substituted protoporphyrins by FAB-MS. The formation of meso, N-diphenylprotoporphyrin IX indicated that the addition of a phenyl radical to the meso-carbon atom of the protoporphyrin ring occurred. Triphenyl and tetraphenyl adducts also indicated the formation of phenyl radicals in the aerobic reaction of phenylhydrazine with oxyhemoglobins. From these results, we suggest that the formation of phenyl radicals and the replacement of heme with phenyl-substituted protoporphyrins cause the destabilization of hemoglobins to induce Heinz bodies and hemolytic anemia with phenylhydrazine.

KEYWORDS: phenylhydrazine, hemoglobin, protoporphyrin, fast-atom-bombardment mass spectrometry (FAB-MS), proton nuclear magnetic resonance (¹H-NMR) spectrometry

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Original Article

Formation of *meso*, N-Diphenylprotoporphyrin IX by an Aerobic Reaction of Phenylhydrazine with Oxyhemoglobins

Akira Nakanishi, Keiko Kinuta, Tadashi Abe,
Kenta Araki, Yumi Yoshida, Shuang Liang, Shun-Ai Li,
Kohji Takei, and Masahiro Kinuta*

Department of Neuroscience, Okayama University Graduate School of
Medicine and Dentistry, Okayama 700-8558, Japan

Administration of phenylhydrazine to rabbits resulted in the denaturation of hemoglobins in erythrocytes, causing the formation of intracellular precipitates known as Heinz bodies, severe hemolytic anemia, and reticulocytosis. To elucidate the molecular mechanism of the destabilization, we allowed human oxyhemoglobins to react aerobically with phenylhydrazine. After treatment with acetic acid/HCl and H₂SO₄/methanol, the chloroform extract contained blue-green pigments of major products accompanied by different minor products. Each product was isolated by column chromatography. By fast-atom-bombardment mass spectrometry (FAB-MS) and proton nuclear magnetic resonance (¹H-NMR) spectrometry, dimethyl esters of N-phenylprotoporphyrin IX and *meso*, N-diphenylprotoporphyrin IX were determined. Other major products also were determined to be dimethyl esters of triphenyl- and tetraphenyl-substituted protoporphyrins by FAB-MS. The formation of *meso*, N-diphenylprotoporphyrin indicated that the addition of a phenyl radical to the *meso*-carbon atom of the protoporphyrin ring occurred. Triphenyl and tetraphenyl adducts also indicated the formation of phenyl radicals in the aerobic reaction of phenylhydrazine with oxyhemoglobins. From these results, we suggest that the formation of phenyl radicals and the replacement of heme with phenyl-substituted protoporphyrins cause the destabilization of hemoglobins to induce Heinz bodies and hemolytic anemia with phenylhydrazine.

Key words: phenylhydrazine, hemoglobin, protoporphyrin, fast-atom-bombardment mass spectrometry (FAB-MS), proton nuclear magnetic resonance (¹H-NMR) spectrometry

Denaturation of oxyhemoglobins by phenylhydrazine in erythrocytes results in the formation of intracellular precipitates known as Heinz bodies [1], severe hemolytic anemia, and reticulocytosis. The molecular mechanism for induction of hemolytic anemia by phenylhydrazine remains to be elucidated. Nitrosobenzene

and phenyl isocyanide, which also induce hemolytic anemia, are ligands in the formation of ferrous complexes of hemoglobins [2]. In the reaction of phenylhydrazine with hemoglobins, however, a complex of phenylhydrazine itself with hemoglobin has not been found. In order to determine the ligand against the heme moiety, we demonstrated previously that the aerobic reaction of chelated protohemin, a synthetic model compound of ferrihemoglobin [3, 4], with phenylhydrazine formed the phenyliron complex of the chelated protohemin [4].

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*Corresponding author. Phone: +81-86-235-7125; Fax: +81-86-235-7126
E-mail: mkinuta@md.okayama-u.ac.jp (M. Kinuta)

Consistently, when phenylhydrazine was allowed to react with myoglobins [5, 6] or methemoglobins [7], the stable phenyl-iron (III) complex of globinproteins was produced.

On the other hand, dimethyl esters of *N*-phenylprotoporphyrin IX (Fig. 1) [8-10] and β -*meso*-phenylbiliverdin IX α (Fig. 1) [8, 9] were isolated as products of the aerobic reaction between phenylhydrazine and hemoglobins. The origin of the *N*-phenyl adduct may be the phenyliron complex of hemoglobin [5, 6]. The formation of the β -*meso*-phenyl adduct suggests the occurrence of both oxidative cleavage of the porphyrin ring at the α -*meso* bridge and the addition of a phenyl radical to the β -*meso*-carbon atom. In the reaction between phenylhydrazine and oxyhemoglobins, however, it remains to be established which is the direct cause of hemoglobin destabilization, the oxidative cleavage or the phenyl-radical formation. Additionally, in the formation of β -*meso*-phenylbiliverdin IX α , determination of which is the first step, the oxidative cleavage or the phenyl substitution, remains to be elucidated. In this work, human oxyhemoglobins were allowed to react aerobically with phenylhydrazine. The chloroform extract of the reaction mixture, after treatment with acetic acid/HCl and H₂SO₄/methanol, yielded various pigments, which were isolated from each other by column chromatography. The dimethyl ester of *N*-phenylprotoporphyrin IX was determined as the product, which was consistent with results reported previously [8-10]. Additionally, a novel blue-green pigment of the major product was isolated, and it

was determined to be a dimethyl ester of *meso*, *N*-diphenylprotoporphyrin IX (Fig. 1) by fast-atom-bombardment mass spectrometry (FAB-MS) and proton nuclear magnetic resonance (¹H-NMR) spectrometry. Dimethyl esters of triphenyl and tetraphenyl substituted protoporphyrins also were isolated as major products. This paper describes the isolation and the structural determination of the phenyl adducts, suggesting that the formation of phenyl radicals and the replacement of heme with phenyl-substituted protoporphyrins are causes for the destabilization of hemoglobin with phenylhydrazine.

Materials and Methods

Chemicals. Phenylhydrazine hydrochloride was recrystallized from ethanol before use. The 3-nitrobenzylalcohol, ²H-chloroform (C²HCl₃), and sodium 3-trimethylsilyl (2,2,3,3-²H₄)propionate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The silica gel 60 (70-230 mesh) for column chromatography and Silica gel 60 thin-layer chromatography (TLC) plate were from Merck (Darmstadt, Germany). Washed erythrocytes from the venous blood of each volunteer were lysed with 3 volumes of water at 4 °C for 40 min and then centrifuged at 12,000 × *g* for 120 min to obtain a clear solution of hemolysate. To remove superoxide dismutase and catalase, the hemolysate was chromatographed by using a CM-cellulose column [11], and dialyzed twice against 0.1 M potassium phosphate buffer, pH 7.4, at 4 °C. The resulting solution of oxyhemoglobin was used for

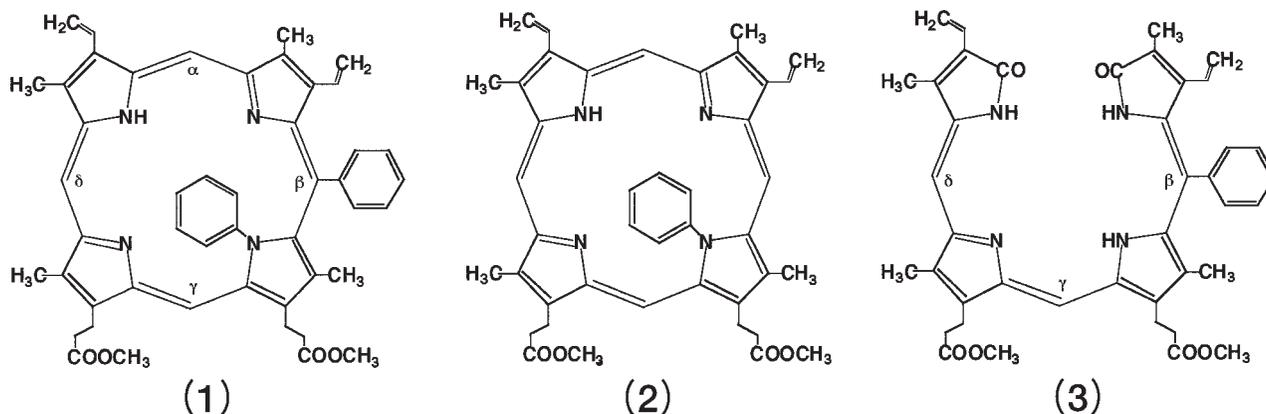


Fig. 1 Structural formulae of dimethyl esters of *meso*, *N*-diphenylprotoporphyrin IX (1), *N*-phenylprotoporphyrin IX (2), and β -*meso*-phenylbiliverdin IX α (3). In (1), the structure shows the β -*meso*-carbon atom to be the possible site of bonding of the phenyl group with the protoporphyrin ring, although the position has not been specified.

reactions with phenylhydrazine. The concentration of the hemoglobin solution was determined as ferrihemoglobin cyanide [12].

Induction of Heinz bodies, anemia, and reticulocytosis with phenylhydrazine.

Female domestic rabbits of 2.5–3 kg body weight were used for the induction of Heinz bodies, anemia, and reticulocytosis. Subcutaneous injection of 55 μ moles of phenylhydrazine in physiological saline per kg of body weight was administered 1, 2, 3, and 4 days after a baseline sample (day 0) of blood was taken. Additional samples of blood were taken 2, 5, and 7 days after the baseline sample. Each packed cell volume (hematocrit value) was determined by using a heparin-coated capillary and centrifugation at 11,000 rpm for 5 min. Each reticulocyte count was based on a count of 1,000 red blood cells, using a light microscopy (LM) equipped with a Miller ocular disc after the cells had been stained with the common solution of 1% (w/v) brilliant cresyl blue in anhydrous ethanol (Pappenheim's staining method) [13]. To determine Heinz bodies, the blood was taken 5 and 7 days after the baseline sample. Heinz bodies also were observed by LM after staining blood cells with the solution of brilliant cresyl blue. Control animals were injected with physiological saline. The statistical significance of differences in the means of each experimental group was calculated with Student's *t*-test. Mean differences were considered to be significant when $P < 0.05$.

Reaction of phenylhydrazine with oxyhemoglobins and extraction of the products.

Experiments were performed in a dark room. Aerobic reaction of phenylhydrazine with oxyhemoglobins was carried out by stirring a 310-ml reaction mixture containing 0.1 M phosphate buffer, pH 7.4, 8.13 mM phenylhydrazine hydrochloride, and 0.813 mM heme of oxyhemoglobins at 25 °C for 70 min without raising air bubbles. After the reaction, we added acetic acid (160 ml) and 12 M HCl (40 ml) in small portions below 4 °C to the reaction mixture, whose color was reddish brown, and then the mixture was stirred slowly at 4 °C for 19 h. Acetic acid (82 ml) and 12 M HCl (21 ml) were further added to the mixture, which was then stirred slowly at 4 °C for 12 h until the solution color changed to greenish blue. The products were extracted 3 times with chloroform, and the extracts were combined, washed twice with water, dried over anhydrous Na₂SO₄, and filtered. The filtrate was evaporated to dryness under the reduced

pressure at 25 °C, and further dried in a vacuum pump for 17 h to obtain a residue. Products in the residue were esterified with H₂SO₄/methanol as described below.

Esterification of products. Methyl esters of the products were prepared by dried methanol in H₂SO₄ essentially as described previously [8]. The residue obtained above was dissolved in 93 ml of dried methanol, and 7 ml of concentrated H₂SO₄ was added slowly to the mixture at 0 °C. After stirring at 4 °C for 24 h in the dark room, 150 ml of cold water was added and then esterified products were extracted twice with chloroform (300 ml) at 4 °C. The extracts were combined, washed with water until the water layer became neutral, dried over anhydrous Na₂SO₄ for 17 h, and filtered. The filtrate was evaporated to dryness, and the residues were further dried in the vacuum pump for 20 h to obtain a dark-green residue. The residue was supplied for the isolation step in Silica gel column chromatography.

Analytical methods. Compounds were determined by physicochemical analyses involving FAB-MS, ¹H-NMR spectrometry, and TLC. FAB-MS with a direct-inlet system was carried out on a Shimadzu 9020-DF gas chromatography-mass spectrometer equipped with a Shimadzu SCAP 1123 data system and a 7240A plotter printer (Hewlett-Packard). The target surface was bombarded by a beam of energetic argon atoms at 5 keV; 3-nitrobenzylalcohol was used as a matrix. ¹H-NMR spectrum was recorded with a JEOL JNM-GX 400 NMR spectrometer in C²HCl₃ with sodium 3-trimethylsilyl (2,2,3,3-²H₄)propionate as an internal standard. TLC was performed on a Silica gel 60 TLC plate by using a solvent system of either benzene/acetone (4:1, v/v) (solvent I) or chloroform/ethanol (22:3, v/v) (solvent II) [8].

Results

Phenylhydrazine induces Heinz bodies, anemia and reticulocytosis.

In order to confirm the effectiveness of phenylhydrazine to induce Heinz bodies and hemolytic anemia, we administered a subcutaneous injection of phenylhydrazine (55 μ mol/kg of body weight) to each rabbit. As shown in Fig. 2, packed cell volume of the baseline sample was $41.2 \pm 3.5\%$. In blood samples taken 2, 5, and 7 days after the baseline sample, packed cell volumes were significantly decreased to 25.3 ± 4.8 , 18.6 ± 5.1 , and $29.5 \pm 6.8\%$ ($P < 0.05$), respectively (Fig. 2). The reticulocyte count of the baseline sample

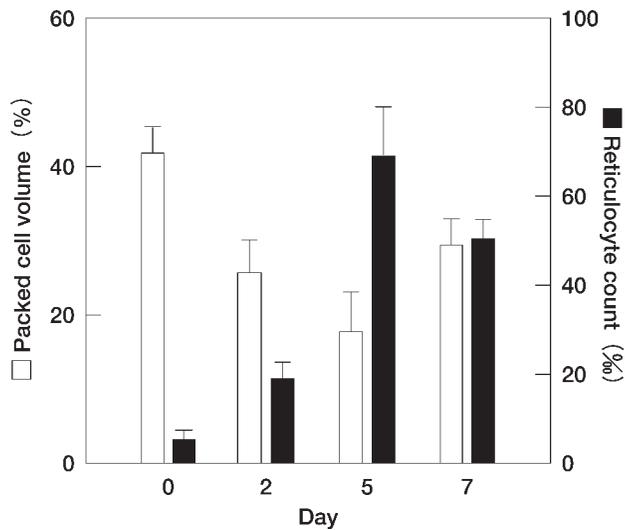


Fig. 2 Packed cell volumes (hematocrit values) and reticulocyte counts in rabbits injected with phenylhydrazine. Subcutaneous injections of 55 μ moles phenylhydrazine in physiological saline per kg of body weight were administered 1, 2, 3, and 4 days after a baseline sample (day 0) of blood was taken. Additional samples of blood were taken 2, 5, and 7 days after the baseline sample. Each packed cell volume (\square) was the mean of three determinations for each sample, and each reticulocyte count (\blacksquare) was based on a count of 1,000 red blood cells. $n = 3$.

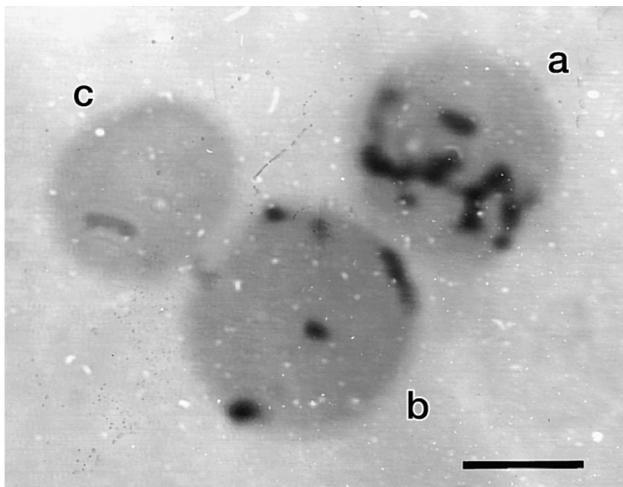


Fig. 3 LM observation of reticulocytes and Heinz bodies formed by the injection of phenylhydrazine in rabbits, administered as described in Fig. 1. The blood was taken 5 days after the baseline sample, and blood cells were stained with the solution of brilliant cresyl blue. a, Reticulocyte; b, A blood cell contains intracellular precipitates known as Heinz bodies; c, Erythrocyte. Bar = 5 μ m.

was $5.8 \pm 1.2\%$, and the count increased significantly to 19.6 ± 4.1 , 68.4 ± 11.9 , and $51.0 \pm 4.4\%$ ($P < 0.05$) of blood samples taken 2, 5, and 7 days, respectively, after the baseline sample (Fig. 2). Intracellular precipitates known as Heinz bodies were observed in the blood sample taken on both day 5 and day 7, accompanied by reticulocytes (Fig. 3). No Heinz bodies were observed in the blood samples from the control animals (data not shown). These results were consistent with the common knowledge that administration of phenylhydrazine to animals induces severe hemolytic anemia due to the denaturation of hemoglobins.

Purification of esterified products by column chromatography. Products of the aerobic reaction between oxyhemoglobins and 10-fold excess of phenylhydrazine were extracted with chloroform and esterified with H_2SO_4 /methanol to form their methyl esters. In the dark room, esterified products were isolated from each other by using a column (3 cm \times 14 cm) of Silica gel G suspended in chloroform. A 2-ml solution of the products (56.3 mg) in chloroform was transferred onto the column and chromatographed at a flow rate of 50 ml/h using a consecutively eluting system with 400 ml of chloroform, 200 ml of acetone/chloroform (5:95, v/v), 300 ml of methanol/chloroform (5:95, v/v), and 1,000 ml of methanol/chloroform (10:90, v/v). Through chromatography, ten bands of pigments due to minor products and 4 pigment bands of major products were found in the column. Each pigment was collected as follows: a light-yellow pigment, an orange-colored pigment, and a mixture of blue pigment/dark green pigment were in 70–90 ml, 120–150 ml, and 270–370 ml of chloroform, respectively, and these were minor products (< 0.5 mg); a blue pigment accompanied by a brown pigment (approximately 0.8 mg) and a green pigment (minor product) were eluted in 70–100 ml and 100–200 ml of 5% (v/v) acetone/chloroform, respectively; a dark-brown pigment and a green pigment was collected in 140–250 ml and 250–300 ml of 5% (v/v) methanol /chloroform, respectively, and these also were minor products (< 0.5 mg); a dark-green pigment and a greenish blue pigment (minor products) were found in 85–100 ml and 100–170 ml of 10% (v/v) methanol/chloroform, respectively. After eluting these minor products, we collected 4 major products of a blue-green pigment (5.0 mg, compound A), a blue-green pigment (6.4 mg, compound B), a blue-green pigment (8.2 mg, compound C), and a green pigment (6.7 mg, compound D) in 170–270 ml, 270–370

ml, 370–470 ml, and 470–770 ml of 10% (v/v) methanol/chloroform, respectively. The eluate containing each pigment was evaporated to dryness under the reduced pressure at 25 °C. To remove the small amount of contaminants, we further purified each major product (compound A–D) using a Silica gel G column (1 cm × 15 cm) with an eluting system of 10% (v/v) methanol/chloroform, and we made physicochemical analyses as described below.

Determinations of dimethyl esters of *N*-phenylprotoporphyrin IX and meso, *N*-diphenylprotoporphyrin IX. The dimethyl ester of *N*-phenylprotoporphyrin IX, after treatment with H₂SO₄/methanol, is the product formed by the aerobic reaction between phenylhydrazine and hemoglobins [8–10]. Since the behavior of compound D in the column chromatography is similar to that of the dimethyl ester [8], compound D was tested by TLC in both solvent I and solvent II. Compound D gave the *R_F* value of 0.02 in solvent I and 0.20 in solvent II. These values were

consistent with those of the dimethyl ester of *N*-phenylprotoporphyrin IX reported previously [8]. In the FAB-mass spectrum of compound D (Fig. 4A), signals at *m/z* 667 (MH⁺, a quasi-molecular ion) and 590 (MH⁺ minus C₆H₅-group) were found, and this supported the formula of C₄₂H₄₂O₄N₄ of the dimethyl ester of *N*-phenylprotoporphyrin IX whose molecular weight (MW) was 666. In the ¹H-NMR spectrum, signals were assigned as follows: (in C²HCl₃; δ in ppm) 3.18 (2*H*, *ortho* protons of *N*-phenyl group), 4.76 (2*H*, *meta* protons of *N*-phenyl group), 5.47 (1*H*, *para* proton of *N*-phenyl group), and 10.12–10.54 (4*H*, *meso* protons). These assignments were also consistent with those of the dimethyl ester of *N*-phenylprotoporphyrin IX reported previously [8]. From these results, compound D was determined to be a dimethyl ester of *N*-phenylprotoporphyrin IX.

Compound C was also determined by FAB-MS and ¹H-NMR. In the FAB-mass spectrum (Fig. 4B), the signal of MH⁺ was found at *m/z* 743, and this allowed

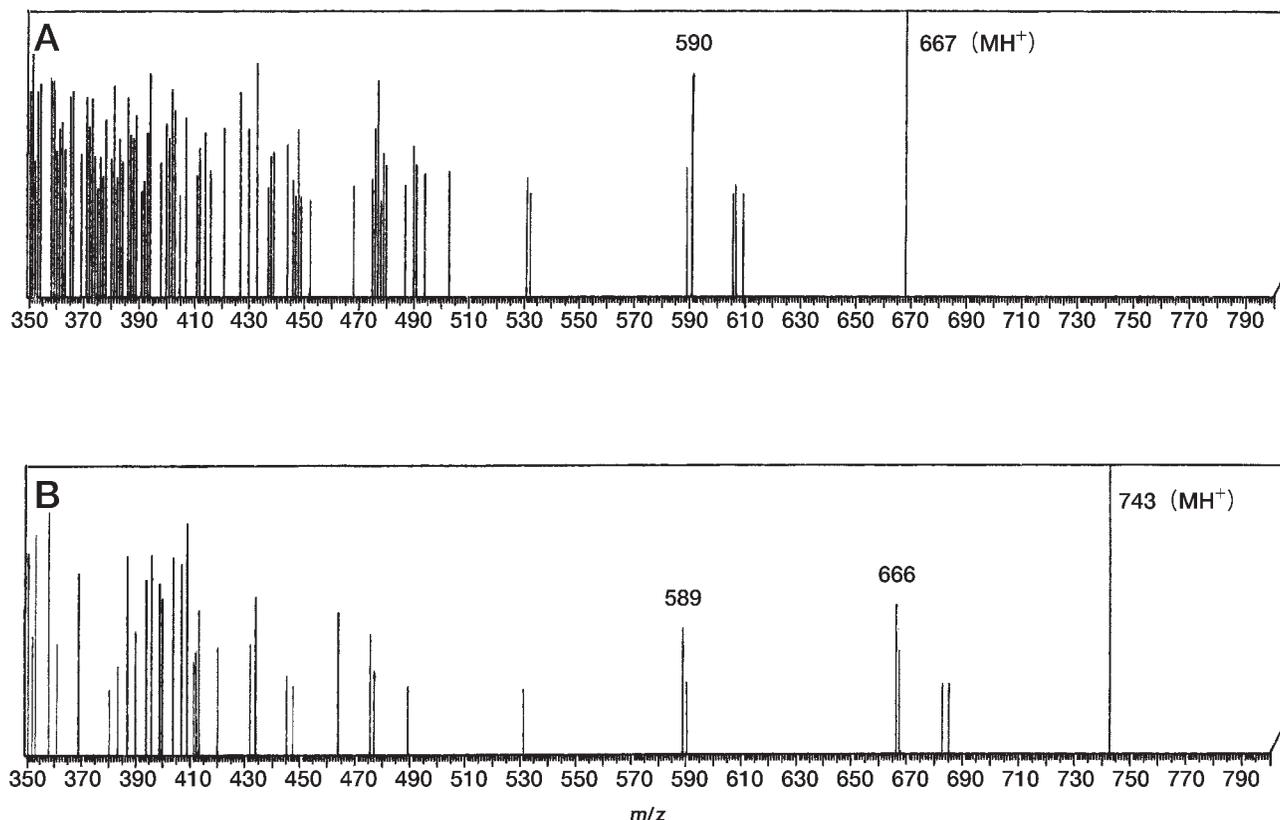


Fig. 4 FAB-mass spectra of *N*-phenylprotoporphyrin IX dimethyl ester (A) and meso, *N*-diphenylprotoporphyrin IX dimethyl ester (B).

us to assume a formula of $C_{48}H_{46}O_4N_4$ (MW, 742). Additionally, signals at m/z 666 (MH^+ minus C_6H_5 -group) and 589 (666 minus C_6H_5 -group) were found, and this allowed us to assume compound C to be diphenylprotoporphyrin IX dimethyl ester. In order to determine the atom bound with each phenyl group, we measured a 1H -NMR spectrum of compound C. In the 1H -NMR spectrum, signals were assigned as follows: (in C^2HCl_3 ; δ in ppm) 3.05–3.25 (4H, $-CH_2-CH_2-CO \times 2$); 3.33 (2H, *ortho* protons of *N*-phenyl); 3.56–3.77 (18H, $-CH_3 \times 4$ and $-OCH_3 \times 2$); 4.20–4.51 (4H, $-CH_2-CH_2-CO \times 2$); 4.78 (2H, *meta* protons of *N*-phenyl group); 5.47 (1H, a *para* proton of *N*-phenyl group); 6.17–6.53 (4H, $-CH=CH_2 \times 2$); 7.47 (5H, protons of *meso*-phenyl group); 8.09–8.23 (2H, $-CH=CH_2 \times 2$); 10.39–10.75 (3H, *meso* protons). As compared with the 1H -NMR spectral data of compound D, we noted that the new signal at δ 7.47 (5H) appeared in the spectrum of compound C and that the number of *meso* protons at δ 10.39–10.75 decreased to 3 because of the disappearance

of one of the *meso*-proton signals [δ , 10.12–10.54 (4H)] found in the spectrum of compound D. These data indicate that one of the phenyl groups bonds to the *meso*-carbon atom of the porphyrin ring. On the other hand, *ortho*-, *meta*-, and *para*-protons of the other phenyl group were found at δ 3.33, 4.78, and 5.47, respectively, consisting with those of the *N*-phenyl group of *N*-phenylprotoporphyrin IX dimethyl ester (compound D) described above. These findings indicated that compound C had 2 phenyl groups on the *meso*-carbon atom and the nitrogen atom of the protoporphyrin ring. From these results, we determined that compound C is the dimethyl ester of *meso*, *N*-diphenylprotoporphyrin IX, although the position of the *meso*-carbon atom on the protoporphyrin ring could not be specified.

Determinations of triphenyl and tetraphenyl substituted protoporphyrins. Compounds A and B of other major products were determined by FAB-MS. In the FAB-mass spectrum of compound A (Fig. 5A), signals were assigned as follows: m/z 895

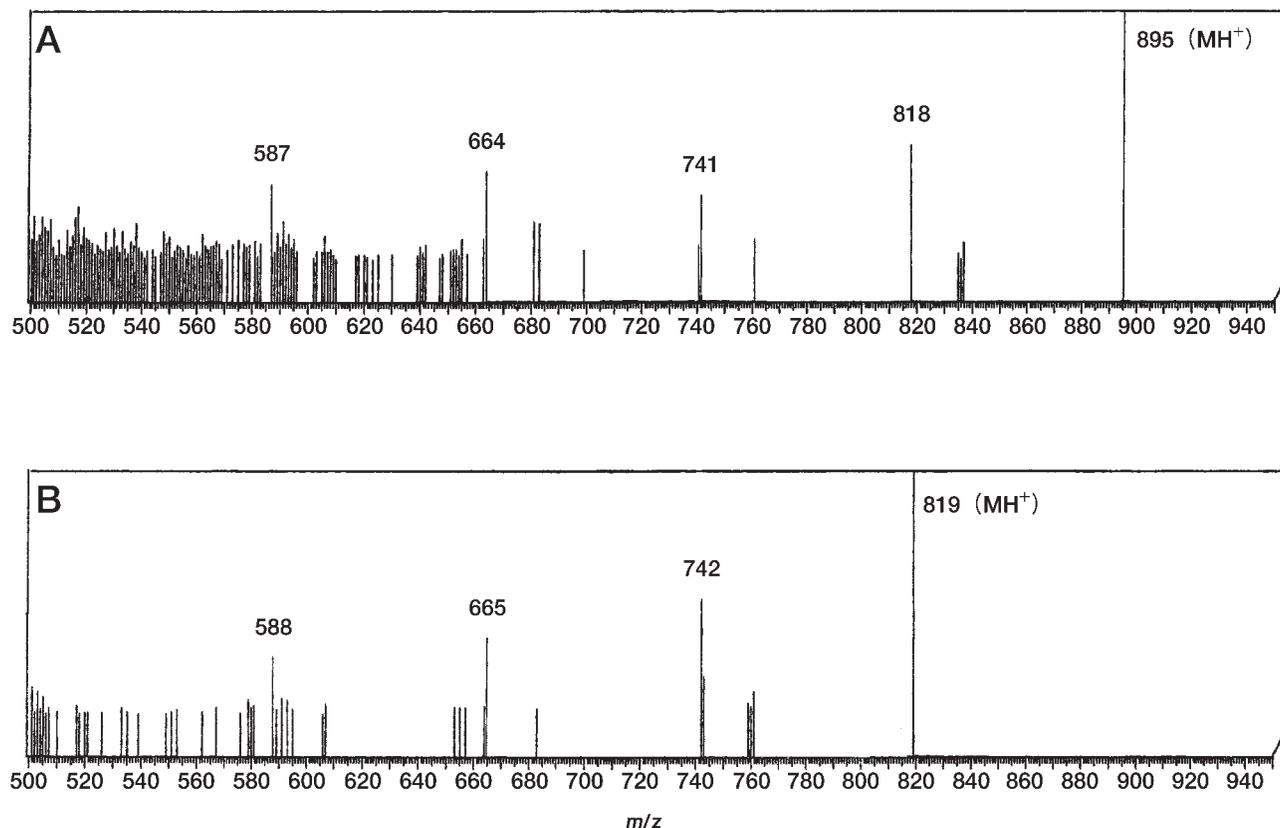


Fig. 5 FAB-mass spectra of dimethyl esters of tetraphenylprotoporphyrin IX (A) and triphenylprotoporphyrin IX (B).

(MH⁺), 818 (MH⁺ minus C₆H₅-group), 741 (818 minus C₆H₅-group), 664 (741 minus C₆H₅-group), and 587 (664 minus C₆H₅-group). On the other hand, in the FAB-mass spectrum of compound B (Fig. 5B), signals were assigned as follows: *m/z* 819 (MH⁺), 742 (MH⁺ minus C₆H₅-group), 665 (742 minus C₆H₅-group), and 588 (665 minus C₆H₅-group). These results suggested that compounds A and B were tetraphenylprotoporphyrin IX dimethyl ester (C₆₀H₅₄O₄N₄, MW = 894) and triphenylprotoporphyrin IX dimethyl ester (C₅₄H₅₀O₄N₄, MW = 818), respectively, although bonding positions of phenyl groups have not been specified because of the lack of ¹H-NMR spectral data for these compounds.

Discussion

We determined that, after treatment with acetic acid/HCl and H₂SO₄/methanol, dimethyl esters of *N*-phenylprotoporphyrin IX and *meso*, *N*-diphenylprotoporphyrin IX are major products of the aerobic reaction of phenylhydrazine with oxyhemoglobins. On the other hand, it has been reported that the dimethyl ester of *β-meso*-phenylbiliverdin IX α (Fig. 1), a blue pigment formed by oxidative cleavage of the porphyrin ring at the α -*meso* bridge, is the product of the aerobic reaction of phenylhydrazine with oxyhemoglobins [8, 9]. Actually, the blue pigment contaminated with the brown pigment was found in the eluate of 5% (v/v) acetone/chloroform as described above. In TLC, the blue pigment gave *R_F* 0.45 in solvent I and 0.90 in solvent II, which were similar to those of *β-meso*-phenylbiliverdin IX α dimethyl ester [8]. In this work, however, no more determination of the pigment was done because of the minor product of the aerobic reaction.

We determined that the novel compound *meso*, *N*-diphenylprotoporphyrin IX was the major product. Regarding the formation of *N*-phenylprotoporphyrin IX, the compound is understood to be an artifact due to the isolation step using acetic acid/HCl and its origin has been proposed to be the phenyliron complex of heme [5, 6]. We demonstrated previously that the anaerobic reaction of phenyldiazene (C₆H₅-N = NH), a compound generated by oxidation of phenylhydrazine [14, 15], with the synthetic model compound of ferrihemoglobin [3, 4] formed a complex of the ferric porphyrin with either phenyldiazenyl anion (C₆H₅-N = NH⁻) or a complex of a ferrous porphyrin with phenyldiazenyl radical (C₆H₅-N = NH[•]) [4]. The complex is theoretically a transient form,

since a phenyliron complex of the chelated protoheme is given immediately when the phenyldiazenyl complex is exposed to oxygen [4]. The phenyliron complex of the chelated protoheme is prepared also by the aerobic reaction of phenylhydrazine with the chelated protohemin, suggesting that the reaction between phenylhydrazine and hemoglobins forms a phenyliron complex of hemoglobin *via* the phenyldiazenyl complex of the ferric porphyrin. In this work, the reaction mixture was treated with acetic acid/HCl in a manner similar to that employed for *N*-phenylprotoporphyrin IX [5, 6, 8]. It is therefore possible that the *N*-phenyl group of the *meso*, *N*-diphenylprotoporphyrin IX molecule arises from the phenyliron complex of heme.

The *meso*-phenyl group was determined by ¹H-NMR spectrometry, and the position of the *meso*-carbon atom could not be specified, unfortunately. Nevertheless, judging from the formation of *β-meso*-phenylbiliverdin IX α , the phenyl group may bond to the *β-meso*-carbon atom of the protoporphyrin ring (Fig. 1). In the aerobic reaction of phenylhydrazine with oxyhemoglobins, phenyl radicals are detected by electron spin resonance spectrometry and the radicals are degraded to benzene at the molar ratio of 5:1 in benzene/heme [10]. The formation of *meso*-phenyl adducts suggested the occurrence of an addition of a phenyl radical to the *meso*-carbon atom. Moreover, in this work, triphenyl and tetraphenyl protoporphyrins were isolated as major products. In the experiment using [U-¹⁴C]-phenylhydrazine [15], the radiolabeled globin protein is determined, although a bonding site of the phenyl group on the protein has not been specified. These findings indicate that phenyl radicals are formed by the aerobic reaction between phenylhydrazine and oxyhemoglobins, and that the phenyl radical functions as an active reactant for the formation of the phenyl adducts. In this work, *meso*-phenylprotoporphyrins were determined to be major products of the aerobic reaction, while there was no detection of unsubstituted biliverdin IX α . It is therefore possible that the initial reaction to form the minor product *β-meso*-phenylbiliverdin IX α is the addition of a phenyl radical to *β-meso*-carbon atom of the porphyrin ring, and then the resulting phenyl adduct, *β-meso*-phenylprotoporphyrin, is cleaved at the α -*meso* bridge.

Phenylhydrazine is used commonly for the induction of experimental anemias. Differences in animal species result in variation in the severity of anemia induced by phenylhydrazine, because a difference in globin molecules

results in a difference in reactivity of phenylhydrazine with hemoglobins [9]. The severity of the induced anemia is influenced also by substituents on the benzene ring of arylhydrazine [15–17]. The severity of anemia induced by *ortho*-substituted arylhydrazines is not as great as that induced by *meta*- and *para*-substituted arylhydrazines [16, 17]. It has been found, by determining changes in the absorption spectra of heme, that the lower effectiveness of *ortho*-substituted arylhydrazines is associated with the lower rate of their initial reaction with hemoglobin [18] and with the ability to form the arylidiazonyliron complex with hemoglobin molecules [16, 17]. The aerobic reaction of *para*-substituted arylhydrazines as well as phenylhydrazine with hemoglobins produce *N*-arylprotoporphyrins after treatment of the reaction mixture with acetic acid/HCl [6, 8]. On the other hand, the aerobic reaction with *ortho*-substituted arylhydrazines does not form *N*-arylprotoporphyrins because of their sterical hindrance of the formation of aryliron complexes and arylidiazonyliron complexes with heme [6, 16, 17]. Moreover, the *ortho*-substitution results in less formation of aryl radicals [6].

Based on these findings, we propose that the reaction of phenylhydrazine with oxyhemoglobins supplies phenyl radicals and the replacement of heme with *meso*-phenyl adducts of protoporphyrin cause the destabilization of hemoproteins, which form Heinz bodies.

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