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Isolation of *N*-Vinylprotoporphyrin IX after Hepatic Cytochrome P450 Inactivation by 3-[(Arylthio)ethyl]sydnone in Chick Embryos Pretreated with Phenobarbital, Glutethimide, Dexamethasone, and β-Naphthoflavone: Differential Inhibition of Ferrochelatase by *N*-Vinylprotoporphyrin Regioisomers

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SUMMARY

Several xenobiotics cause hepatic porphyrin accumulation through mechanism-based inactivation of cytochrome P450 (P450) and heme alkylation. Loss of iron from the alkylated heme results in formation of an *N*-alkylporphyrin, which is a potent inhibitor of ferrochelatase. *N*-Vinylprotoporphyrin IX (*N*-vinylPP) was identified in chick embryo liver after *in ovo* administration of 3-[(arylthio)ethyl]sydnone (TTMS). Pretreatment of chick embryos with β-naphthoflavone, which causes a 90-fold increase in P450 1A levels, did not increase the formation of *N*-vinylPP after TTMS administration, showing that the heme moiety of P450 1A does not contribute to the formation of *N*-vinylPP. Increased amounts of *N*-vinylPP were isolated from dexamethasone-, phenobarbital-, and glutethimide-pretreated chick embryos, and it is possible that P450 2H and/or a P450 3A-like isozyme contributes to the formation of *N*-vinylPP. The ring B-substituted (N_B) regioisomer of *N*-vinylPP constituted the lowest percentage of the total regioisomers (9–13%) in untreated and drug-induced chick embryos, thus supporting the concept that ring B of heme is occluded by a protein residue in the P450 active site. Previously, the finding that the N_B regioisomer of *N*-ethylprotoporphyrin IX had one fifth the potency of the ring A-substituted (N_A) regioisomer as a ferrochelatase inhibitor led to a proposal that an A—C ring tilt was important in *N*-alkylprotoporphyrins for ferrochelatase inhibition. The finding in the present study that the N_A and N_B regioisomers of *N*-vinylPP are equipotent does not support the above proposal. The ring C-substituted (N_C) and ring D-substituted (N_D) regioisomers of *N*-vinylPP had low potency.

Xenobiotics that disrupt heme biosynthesis and cause porphyrin accumulation are referred to as porphyrinogenic compounds. Rat hepatic P450 (EC 1.14.14.1) isozymes 1A1, 2C6, 2C11, 3A1, and 3A2 and chick embryo hepatic P450 1A, 2H, and 3A-like isozymes are targets for mechanism-based inactivation by the porphyrinogenic compound 4-ethyl DDC (Fig. 1a) (1–8). Mechanism-based inactivation is the result of prosthetic heme *N*-alkylation of P450 by an ethyl radical generated during the metabolism of 4-ethyl DDC by P450 isozymes (9). After heme *N*-ethylation, iron is released from *N*-ethylheme to form a mixture of four regioisomers of *N*-ethylprotoporphyrin IX (*N*-ethylPP) (Fig. 1c). The N_A regioisomer of *N*-ethylPP isolated from rat liver was found to be 5 times more potent an inhibitor of ferrochelatase (EC 4.99.1.1) than was the N_B regioisomer (10). The N_C and N_D regioisomers had low potency as inhibitors of ferrochelatase (11).

According to one proposed mechanism, *N*-alkylPPs inhibit ferrochelatase by serving as transition state analogues for the iron-insertion step (12). X-ray crystallography shows that the *N*-alkyl group-bearing pyrrole ring and the pyrrole ring opposite to the *N*-alkyl group are tilted out of planarity in opposite directions (13). We suggested that this tilting reflects the normal conformational change required for the insertion of iron into the protoporphyrin IX ring by ferrochelatase and that the greater inhibitory activity of the N_A compared with the N_B regioisomer is due to the fact that the normal mechanism for ferrochelatase-catalyzed iron insertion has preference for an A—C ring tilt over a B—D ring tilt (10).

Rat hepatic P450 1A and 3A and chick embryo hepatic

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ABBREVIATIONS: P450, cytochrome P450; TTMS, 3-[(arylthio)ethyl]sydnone; 4-ethyl DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; *N*-vinylPP, *N*-vinylprotoporphyrin IX; *N*-alkylPP, *N*-alkylprotoporphyrin IX; *N*-ethylPP, *N*-ethylprotoporphyrin IX; DEX, dexamethasone; βNF, β-naphthoflavone; PB, phenobarbital; GLUT, glutethimide; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

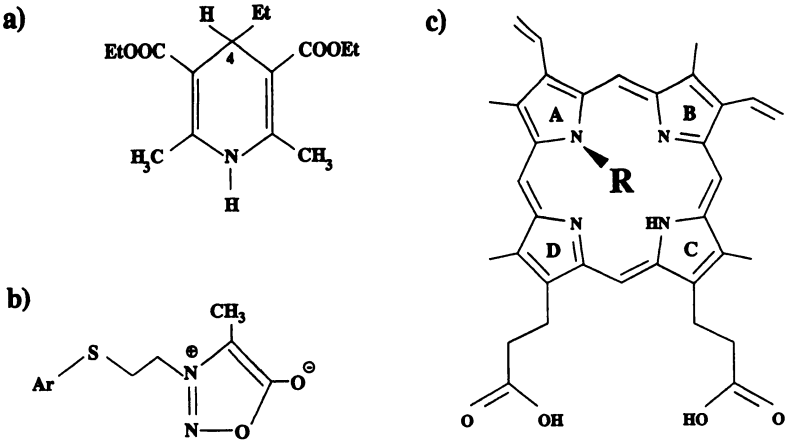

Fig. 1. Structure of 4-ethyl (*Et*) DDC (a), TTMS (b), and the N_A regioisomer of an *N*-alkylPP (c). The other regioisomers have the *N*-alkyl group located on the B (N_B), C (N_C), and D (N_D) pyrrole rings. For *N*-ethylPP, *R* = —CH_2CH_3. For *N*-vinylPP, *R* = —CH=CH_2.

P450 1A, 2H, and 3A-like isozymes have been shown to be targets for mechanism-based inactivation by the porphyrinogenic sydnone TTMS (Fig. 1b) (7, 8, 14–16). After mechanism-based inactivation of P450 isozymes in rat liver by TTMS, a mixture of N_A, N_B, N_C, and N_D regioisomers of *N*-vinylPP were isolated and identified, and it was assumed that because TTMS caused protoporphyrin accumulation in rodents and dogs, this mixture of *N*-vinylPP regioisomers was responsible for ferrochelatase inhibition (14). However, the ferrochelatase-inhibitory activity of *N*-vinylPP regioisomers has not been studied. It has been suggested (17) that individual P450 isozymes may give rise preferentially to a different regioisomer of *N*-alkylPP.

The objectives of the current study were 1) to determine whether *N*-vinylPP is formed in chick embryo liver after the *in ovo* administration of TTMS to 19-day-old chick embryos; 2) to determine the effect of P450 isozyme inducers on the formation of *N*-vinylPP (for this purpose, TTMS was administered *in ovo* to chick embryos pretreated with βNF, DEX, PB, or GLUT); 3) to compare the regioisomer composition of *N*-vinylPP formed in the livers of untreated and βNF-, DEX-, PB-, and GLUT-pretreated chick embryos; 4) to compare the potency of the N_A, N_B, N_C, and N_D regioisomers of *N*-vinylPP as inhibitors of chick embryo hepatic ferrochelatase; and 5) to determine whether support could be provided for the hypothesis that the normal mechanism for ferrochelatase-catalyzed iron insertion has preference for an A—C ring tilt over a B—D ring tilt. If this hypothesis is correct, then we anticipated that the N_A regioisomer of *N*-vinylPP would have similar potency to the N_A regioisomer of *N*-ethylPP and be more potent than the N_B regioisomer of *N*-vinylPP.

Experimental Procedures

Materials. TTMS was obtained from Colour Your Enzyme (Bath, Ontario, Canada). PB, HPLC-grade tetrahydrofuran (stabilized with 250 ppm butylated hydroxytoluene), hexane, methanol, acetone, and dichloromethane were purchased from British Drug Houses (Toronto, Ontario, Canada); βNF from Aldrich Chemical Co. (Milwaukee, WI); DEX and GLUT from Sigma Chemical Co. (St. Louis, MO); and Analtech silica gel G TLC plates (20 × 20 cm) and Whatman Partisil 10 PAC HPLC columns (4.6 mm i.d. × 25 cm) from Mandel Scientific Co. Ltd. (Guelph, Ontario, Canada).

Animals. Fertilized eggs of the white leghorn strain were obtained from Archer's Poultry Farm (Brighton, Ontario, Canada). Eggs were stored at 4° for ≤9 days before incubation in a Humidaire Incubator (New Madison, OH) at 37° with a humidity of 86%. Embryo age was considered to be the number of days from the onset of incubation.

Induction of chick embryo hepatic P450 and isolation of *N*-vinylPP from chick embryo liver. Chick embryos received no treatment or were injected with an inducer of P450 isozymes through a small hole made in the egg shell above the air sac with a 1-in. 20-gauge needle and a 1-ml Tuberculin syringe. PB was administered in 0.1 ml of deionized water (6 mg/egg/day for 2 days), βNF in 0.1 ml of DMSO (2 mg/egg for 1 day), DEX in 0.1 ml of DMSO (5 mg/egg for 1 day), and GLUT in 0.1 ml of DMSO (4 mg/egg for 1 day) (7, 8, 18) so that all of the chick embryos had been incubated for 19 days at the end of the treatment period. After administration of the inducer, the hole in the egg shells was sealed with cellotape, and the eggs were returned to the incubator.

At 24 hr after the final inducer treatment, the chick embryos were injected with a single dose of TTMS in 0.1 ml of DMSO (4 mg/egg). At 4 hr later, the 19-day-old chick embryos were killed by decapitation, and their livers were removed and rinsed in ice-cold 1.15% KCl. Livers from the same induction group were pooled and homogenized in ice-cold 5% H_2SO_4/methanol (5–10 ml/liver) and stored in the dark at 4° overnight. The *N*-vinylPP dimethyl ester mixture was filtered, diluted with an equal volume of deionized water, and extracted with dichloromethane (3 × 2 ml/liver) in a separatory funnel. The dichloromethane solution was washed successively with 5% sodium bicarbonate (5 ml/liver) and water (twice at 5 ml/liver) and then dried over anhydrous sodium sulfate for 1 hr. The sodium sulfate was removed by filtration, zinc acetate (25 μmol) in methanol (2 ml) was added to the dichloromethane solution to form Zn *N*-vinylPP dimethyl ester, and the solution was evaporated to dryness (14).

Purification of Zn *N*-vinylPP by TLC. The zinc-complexed *N*-vinylPP dimethyl esters were separated into the N_A-plus-N_B and N_C-plus-N_D regioisomer pairs by two consecutive TLC procedures, as described previously (19). The Zn *N*-vinylPP residue was redissolved in dichloromethane, applied to an Analtech silica gel G TLC plate (2000 μm), and developed in dichloromethane/methanol (20:3) for 50–60 min. A single green band (R_F = 0.68–0.74) that fluoresced red under UV light was eluted from the plate with acetone and evaporated to dryness. The residue was redissolved in dichloromethane, applied to an Analtech silica gel G plate (1000 μm), and developed in dichloromethane/acetone (5:1) for 50–60 min. Two green bands (R_F = 0.67–0.72 and 0.75–0.80) that fluoresced red under UV light were eluted separately from the plate with acetone and evaporated to dryness. Dichloromethane was added, and the electronic absorption spectra were determined with a Hewlett Packard 8451A diode array spectrophotometer. The concentration of the Zn *N*-vinylPP was determined with use of the molar extinction coefficient for Zn *N*-methylprotoporphyrin (124,000 M^{-1} cm^{-1} at 432 nm) (20). In previous studies, *N*-methylPP was added in a range of concentrations to rat livers and homogenized with ice-cold 5% H_2SO_4/methanol before

extraction and purification by TLC. The recovery was found to be 25% (21).

Separation of the *N*-vinylPP regioisomer pairs by HPLC. The *N*-vinylPP dimethyl esters were separated into individual regioisomers by two consecutive HPLC procedures, as described previously (14, 19, 20). The Zn *N*-vinylPP pairs were partially purified by HPLC on a Partisil 10 PAC column and eluted with a 20-min linear gradient (0–100%) of methanol in hexane/tetrahydrofuran (1:1) at a flow rate of 1 ml/min. Absorption was monitored at 432 nm. Fractions with the characteristic spectrum of a Zn *N*-alkylPP (14, 20) were pooled, and the zinc cation was removed by treatment with 5% H_2SO_4/methanol (22). The Zn-free *N*-vinylPP regioisomer pairs were separated into individual regioisomers on a second Partisil 10 PAC column. The regioisomers were eluted isocratically with hexane/tetrahydrofuran/methanol/water (48.45:48.45:3:0.1) at a flow rate of 0.5 ml/min. Absorption was measured at 420 nm. The regioisomers were collected separately, and their electronic absorption spectra were compared with those previously obtained in the rat (14, 20).

Ferrochelatase-inhibitory activity. The four regioisomers of *N*-vinylPP dimethyl ester were converted to free acids by hydrolysis in 500 μl of 6.0 N hydrochloric acid overnight in the dark at room temperature (22). After removal of the hydrochloric acid under a stream of nitrogen, the *N*-vinylPPs were dissolved in 95% ethanol, and a series of dilutions were prepared. Aliquots (0.1 ml in 95% ethanol) were transferred to the sidearm of Thunberg tubes. Control tubes received 0.1 ml of 95% ethanol.

Details of the cell culture technique have been reported (10, 23). Ferrochelatase-inhibitory experiments were conducted in chick embryo hepatocyte homogenates because this is a standard method that we used in earlier studies and allows comparison with extensive previous studies. The 18-day-old chick embryo liver cells were maintained in 10-cm-diameter plastic Petri dishes containing 15 ml of serum-free Waymouth MD 705/1 medium supplemented with penicillin G sodium (60 mg/liter), streptomycin sulfate (100 mg/liter), sodium bicarbonate (2.24 g/liter), insulin (1 mg/liter), and L-thyroxine sodium pentahydrate (1 mg/liter). After a 24-hr incubation at 37°, the medium was replaced. After an additional 24-hr incubation, the cells were harvested with a buffer containing 0.25 M sucrose, 0.05 M Tris·HCl, and 1.0 mM EDTA, pH 8.2. The cells were centrifuged for 5 min at 500 × *g*, washed with the sucrose-Tris buffer (1.0 ml/Petri dish), and homogenized in 0.6 ml/dish 0.02 M Tris·HCl buffer, pH 8.2. Aliquots of the homogenized cells (0.9 ml) were transferred to the sidearm of the Thunberg tubes containing the *N*-vinylPP (0.1 ml).

The body of the Thunberg tubes contained mesoporphyrin IX (120 nmol), 1% (w/v) Tween 80 (0.3 ml), 95% ethanol (0.3 ml), 0.2 M Tris·HCl buffer, pH 8.2 (1.5 ml), 0.2 M dithioerythritol (60 μl), and 1.0 mM ferrous sulfate (120 μl). The sidearm was connected to the body of the Thunberg tube, and the atmosphere was made anaerobic by alternating vacuum with nitrogen several times. After a 5-min preincubation in a 37° water bath, the contents of the sidearm were tipped into the body of the Thunberg tubes for a 10-min incubation at 37°. The reaction was terminated by the addition of 0.4 M iodoacetamide (0.5 ml) to the tubes.

The amount of mesoheme formed was determined by using the reduced versus oxidized spectrum of the pyridine mesohemochromogen (24–26), recorded from 500 to 560 nm on a Hewlett Packard 8451A diode array spectrophotometer. Ferrochelatase activity was expressed as nmol mesoheme/mg protein/10 min. Protein was measured according to the method of Lowry *et al.* (27).

Results and Discussion

Identification of *N*-vinylPP in chick embryo liver after the *in ovo* administration of TTMS. The zinc-complexed *N*-alkylPP isolated from 15–41 pooled chick embryo livers was identified as Zn *N*-vinylPP in the following manner. The crude Zn *N*-alkylPP was separated by TLC into two fluorescing green bands. The band with an R_F value of 0.75–0.80 was tentatively identified as a mixture of N_A and N_B regioisomers, and the band with an R_F value of 0.67–0.72 was tentatively identified as a mixture of the N_C and N_D regioisomers (19). This identification was supported by the characteristics of the electronic absorption spectra of the two TLC bands. Thus, the band with an R_F of 0.75–0.80 had a shoulder on the Soret band at 432 nm, whereas the band with an R_F of 0.67–0.72 did not have this shoulder on the Soret band. The shoulder on the Soret band has previously been shown to be diagnostic for the N_A and N_B regioisomers of *N*-alkylPP (14, 20). After purification of the Zn *N*-alkylPP by HPLC, the zinc was removed and the *N*-alkylPP was separated into the individual N_A, N_B, N_C, and N_D regioisomers. The order of elution from the column that was observed, i.e., N_B, N_A, N_C, and N_D regioisomers, is in accord with that previously reported (14, 20). A typical separation of regioisomers is shown in Fig. 2. In a previous study, TTMS was administered *in vivo* to PB-pretreated rats, and the *N*-alkylPP isolated was identified as *N*-vinylPP through a combination of electron absorption, NMR, and mass spectroscopic methods (14). The first objective of the current study was to determine whether *N*-vinylPP is formed in chick embryo liver after the *in ovo* administration of TTMS to 19-day-old chick embryos. On the basis of the R_F values in TLC, the retention times in HPLC, and the electronic absorption spectra of the Zn *N*-alkylPP and the Zn-free *N*-alkylPP regioisomers, we concluded that we had isolated authentic regioisomers of *N*-vinylPP. In a previous study (28), 100 PB-pretreated chick embryos were used to isolate 0.81 nmol/g wet weight liver of *N*-ethylPP after the administration of 4-ethyl DDC (15 mg/egg). In the current study, we succeeded in using a single PB-pretreated chick embryo to isolate 15.12 nmol/g wet weight liver of *N*-vinylPP after the administration of TTMS (4 mg/egg). Clearly, therefore, TTMS results in the conversion of greater quantities of the heme moiety of chick embryo hepatic P450 to *N*-alkylPPs than does 4-ethyl DDC.

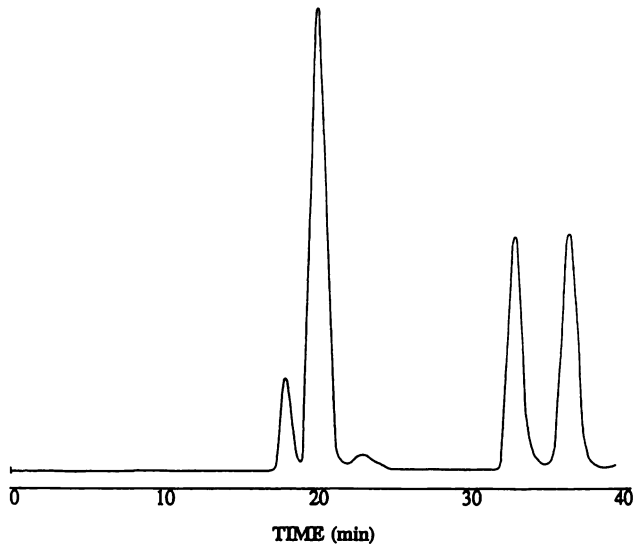

Fig. 2. HPLC analysis of *N*-vinylPP dimethyl ester isolated from untreated chick embryos after the administration of TTMS. A Partisil 10 PAC column (4.6 × 250 mm), eluted (0.5 ml/min) with hexane/tetrahydrofuran/methanol/water (48.25:48.25:3.0:0.1), and a variable wavelength detector set at 420 nm were used for the analysis. The order of elution of the regioisomers is N_B, N_A, N_C, and N_D.

Effect of P450 isozyme inducers on *N*-vinylPP formation after the *in ovo* administration of TTMS to chick embryos. In a previous study (7), we demonstrated mechanism-based inactivation of P450 by TTMS when added to chick embryo hepatic microsomes. Loss of P450 was 31% in untreated, 36% in βNF-treated, 42% in DEX-treated, and 34% in PB-treated hepatic microsomes. After the *in ovo* administration of TTMS to chick embryos, hepatic P450 levels were found to decrease by 31% in untreated, 68% in βNF-treated, 37% in DEX-treated, and 82% in PB-treated and GLUT-treated chick embryos (8). Chick embryo hepatic P450 1A-, 2H-, and 3A-like isozymes are targets for mechanism-based inactivation by TTMS (7, 8). The second objective of the current study was to determine the effect of P450 isozyme inducers on the formation of *N*-vinylPP in the chick embryo. Chick embryo hepatic P450 isozymes were induced with βNF, DEX, PB, or GLUT. At 24 hr later, the chick embryo received TTMS, and after 4 hr, Zn *N*-vinylPP dimethyl ester was isolated and purified by TLC. Electronic absorption spectra were used to quantify the amount of Zn *N*-vinylPP (nmol/g wet weight liver) that was formed in untreated and βNF-, DEX-, PB-, and GLUT-pretreated chick embryo liver after the administration of TTMS. We found that βNF pretreatment did not significantly increase the formation of Zn *N*-vinylPP over untreated levels (2.09 nmol/g liver) (Fig. 3). It has been reported previously that βNF induces P450 1A activity by 90-fold in the chick embryo (7). Because βNF pretreatment did not cause an increase in the formation of *N*-vinylPP over untreated levels, it is clear that P450 1A is not the source of the P450 for the formation of *N*-vinylPP in the chick embryo. It is of interest that TTMS-induced loss of P450 in the hepatic microsomes from βNF-treated chick embryos was 68% compared with 31% in untreated chick embryos (8). Thus, the failure to observe an enhancement of *N*-vinylPP formation in βNF-treated embryos cannot be explained by resistance of the P450 in βNF-treated embryos to destruction by TTMS. It is likely that destruction of P450 in the βNF-treated chick embryo must proceed by a mechanism that does not result in *N*-vinylPP formation.

It has been shown previously that erythromycin *N*-demethylase and testosterone 6β-hydroxylase, enzyme activities associated with the cytochrome P450 3A family, were induced by DEX and/or PB in chick embryo liver (7, 29, 30). In addition, it has been shown that DEX and PB induced a P450 3A-like protein in chick embryo liver, as detected by monoclonal antibodies to rat P450 3A (29). However, in contrast to the rat, in which DEX is a much more effective inducer of P450 3A than PB, in the chick embryo PB is a more effective inducer of a P450 3A-like isozyme than is DEX (7). In the current study, DEX and PB caused a 2.1- and 6.8-fold increase, respectively, in the formation of *N*-vinylPP over untreated levels. Therefore, a P450 3A-like isozyme may play a role in the formation of *N*-vinylPP in the chick embryo. Further studies with a purified chick embryo P450 3A-like isozyme will be necessary to confirm this. It has been reported that PB and GLUT produce a marked induction of P450 2H1/2 in the chick embryo (31). We found a 6.8- and 10.2-fold increase in the formation of *N*-vinylPP over untreated levels after PB and GLUT pretreatment, respectively (Fig. 3). Therefore, it is possible that P450 2H1/2, or an unidentified PB- and GLUT-inducible P450 isozyme, contributes significantly to the formation of *N*-vinylPP in the chick embryo.

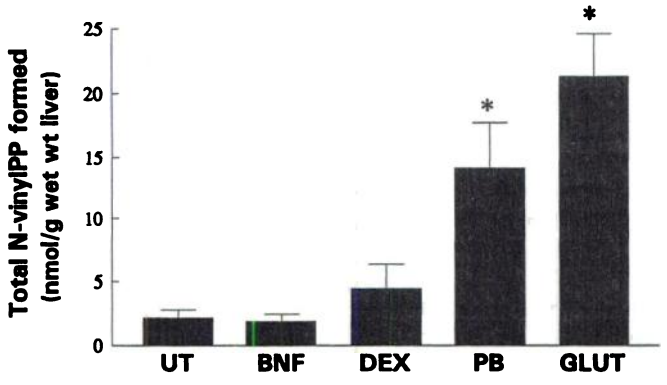

Fig. 3. Effect of *in ovo* administration of TTMS (4 mg/egg) on the formation of *N*-vinylPP in untreated (*UT*) and βNF (*BNF*)-, DEX-, PB-, and GLUT-pretreated chick embryos. Values represent the mean ± standard deviation of determinations from three to five groups of 15–41 chick embryo livers per group. *, Significantly different ($p \leq 0.05$) from untreated chick embryo liver, as determined with a randomized-design one-way analysis of variance and Newman-Keuls test.

Comparison of the regioisomer composition of *N*-vinylPP formed in untreated and βNF-, DEX-, PB-, and GLUT-pretreated chick embryos. De Matteis *et al.* (17) obtained evidence indicating that individual P450 isozymes may give rise preferentially to a different regioisomer of *N*-alkylPP. Thus, the administration of 4-ethyl DDC to PB-pretreated rats resulted in the identification of the N_A regioisomer of *N*-ethylPP as the major regioisomer compared with the N_C regioisomer in untreated rats. When 4-ethyl DDC was administered to βNF-pretreated rats, the N_D regioisomer was identified as the major regioisomer. These investigators interpreted these results as indicating that the main PB-inducible P450 in rat liver produces preferentially regioisomer N_A, whereas the βNF-inducible P450 produces regioisomer N_D. It was suggested that the apoprotein of a P450 may either direct the alkyl group liberated from metabolism of 4-ethyl DDC on to one of the four pyrrole nitrogens or cause a change in the state of heme, leading to increased reactivity of a different pyrrole nitrogen.

The third objective of the current study was to compare the regioisomer composition of *N*-vinylPP formed in the livers of untreated and βNF-, DEX-, PB-, and GLUT-pretreated chick embryos. We found no significant differences in *N*-vinylPP regioisomer composition among untreated and βNF- and DEX-pretreated chick embryos (Fig. 4a). However, amounts of all four regioisomers were found to be significantly greater in the PB- and GLUT-pretreated than in the untreated chick embryos (Fig. 4a). It was therefore concluded that unlike De Matteis *et al.* (17), who assigned preferential *N*-ethylPP regioisomer formation to PB- and βNF-inducible P450s, we are unable to assign preferential regioisomer formation to P450s induced by specific inducers in the chick embryo. The N_A regioisomer constituted a significantly smaller percentage of the total *N*-vinylPPs in βNF-, DEX-, PB-, and GLUT-pretreated chick embryos than in untreated chick embryos, where it constituted 56% of the *N*-vinylPP regioisomers (Fig. 4b). The N_D regioisomer constituted a significantly greater percentage of the total *N*-vinylPPs in PB- and GLUT-pretreated chick embryos (34% and 40%, respectively) than in untreated chick embryos, where it constituted 17% of the *N*-vinylPP regioisomers (Fig. 4b).

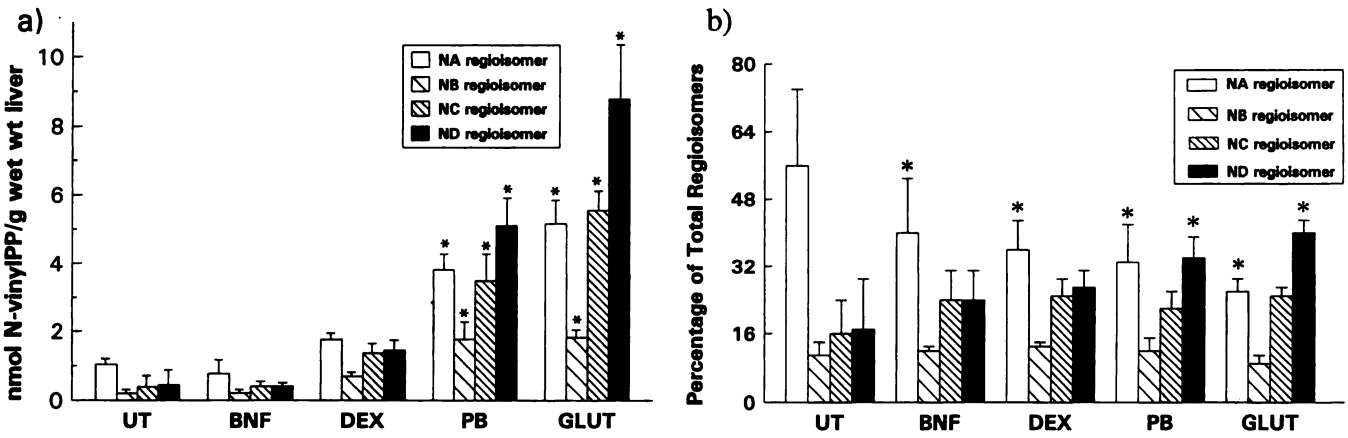

Fig. 4. Regioisomer composition of *N*-vinylPP formation after the *in ovo* administration of TTMS (4 mg/egg) to untreated (*UT*) and βNF (*BNF*)-, DEX-, PB-, and GLUT-pretreated chick embryos. a, Amount of each regioisomer formed. b, Regioisomer formation as a percentage of total regioisomers. Values represent the mean ± standard deviation of determinations from four to six groups of 15–41 chick embryo livers per group. *, Significantly different ($p \leq 0.05$) from the corresponding regioisomer of *N*-vinylPP from untreated chick embryo liver, as determined with a randomized-design one-way analysis of variance and Newman-Keuls test.

Swanson *et al.* (32) performed a topological analysis of the active sites of rat hepatic P450 1A1, 2B1, 2B2, and 2E1 by *in situ* rearrangement of phenyl/iron complexes. These researchers (32) were able to isolate only two *N*-phenylprotoporphyrin IX regioisomers with the *N*-phenyl group on pyrrole rings A and D. They concluded that the active sites of these P450 isozymes "had similar structural motifs with pyrrole rings B and C at least partially masked by protein residues and an open region above pyrrole rings A and D" (32). Previously, Kunze *et al.* (33) studied the regiospecificity of cytochrome P450 heme *N*-alkylation by olefins and acetylenes and concluded that pyrrole ring B of the heme is partially masked by protein residues. It is of interest to consider our data in the light of the above studies. The N_B regioisomer (Fig. 4b) constituted the lowest percentage of regioisomers (9–13%) and was present in the smallest quantity in untreated and drug-induced chick embryos (Fig. 4a). These observations support the concept that ring B of heme is occluded by a protein residue or residues in the active site of P450. The N_C regioisomer constituted 16–25% of the total *N*-vinylPP regioisomers, and considerably larger amounts of the N_C than of the N_B regioisomer were found in several of the induction groups (Fig. 4a). Thus, no support could be provided by our studies for a degree of masking of pyrrole ring C comparable with that of pyrrole ring B.

Comparison of the potencies of N_A, N_B, N_C, and N_D regioisomers of *N*-vinylPP as inhibitors of chick embryo hepatic ferrochelatase. It has been suggested that *N*-alkylPPs inhibit ferrochelatase by serving as transition state analogues for the iron-insertion step (12). X-ray crystallography shows that the *N*-alkyl group-bearing pyrrole ring and the pyrrole ring opposite to the *N*-alkyl group are tilted out of planarity in opposite directions (13). We suggested that this tilting reflects a normal conformational change required for the insertion of iron into the protoporphyrin IX ring by ferrochelatase and that the greater inhibitory activity of the N_A regioisomer of *N*-ethylPP compared with that of the N_B regioisomer of *N*-ethylPP is due to the fact that the normal mechanism for ferrochelatase-catalyzed iron insertion has preference for an A—C ring tilt over a B—D ring tilt (10). We thus anticipated that the N_A regioisomer of *N*-vinylPP would have a higher potency than the N_B regioisomer of *N*-vinylPP. The fourth objective of the current study was to compare the potencies of the N_A, N_B, N_C, and N_D regioisomers of *N*-vinylPP as inhibitors of chick embryo hepatic ferrochelatase and to determine whether support could be provided for the hypothesis that the normal mechanism for ferrochelatase-catalyzed iron insertion has preference for an A—C ring tilt over a B—D ring tilt. The data in Fig. 5a show that the N_A and N_B regioisomers of *N*-vinylPP from chick embryo livers are equipotent as inhibitors of chick embryo liver ferrochelatase activity. The EC_{50} values for the N_A and N_B regioisomers are 0.030 and 0.035 μM, respectively (Table 1 and Fig. 5a), and the EC_{75} values are 0.013 and 0.012 μM, respectively (Fig. 5a). Because of the low potency of the N_C and N_D regioisomers, EC_{50} values could not be obtained; instead, EC_{75} values of 2.3 and 1.0 μM were obtained for the N_C and N_D regioisomers, respectively (Fig. 5b). Thus, the N_D regioisomer of *N*-vinylPP is 2.3 times more potent an inhibitor of ferrochelatase activity than the N_C regioisomer (Fig. 5b), a difference to which we do not attach any biological significance. The equipotent N_A and N_B regioisomers of *N*-vinylPP are 184- and 80-fold more potent inhibitors of ferrochelatase activity than the N_C and N_D regioisomers, respectively. This result is in accord with previous results that show that the N_A and N_B regioisomers of *N*-alkylPPs, with the exception of *N*-methylprotoporphyrin (34), are markedly more potent inhibitors of chick embryo ferrochelatase than the N_C and N_D regioisomers (11, 26).

For *N*-ethylPP isolated from rat liver, the N_A regioisomer was 5-fold more potent an inhibitor of ferrochelatase than the N_B regioisomer (10). The equipotent N_A and N_B regioisomers of *N*-vinylPP isolated from chick embryo liver in the current study had a similar potency as the N_B regioisomer of *N*-ethylPP isolated from rat liver (Table 1). To ensure the validity of the comparison between the ferrochelatase-inhibitory activity that we measured with *N*-vinylPP regioisomers in this study with that of the N_A and N_B regioisomers of *N*-ethylPP measured previously (10), we included with our current study a single sample of the synthetic N_A and synthetic N_B regioisomers of *N*-ethylPP. The ferrochelatase-inhibitory potency measured with these synthetic regioisomers

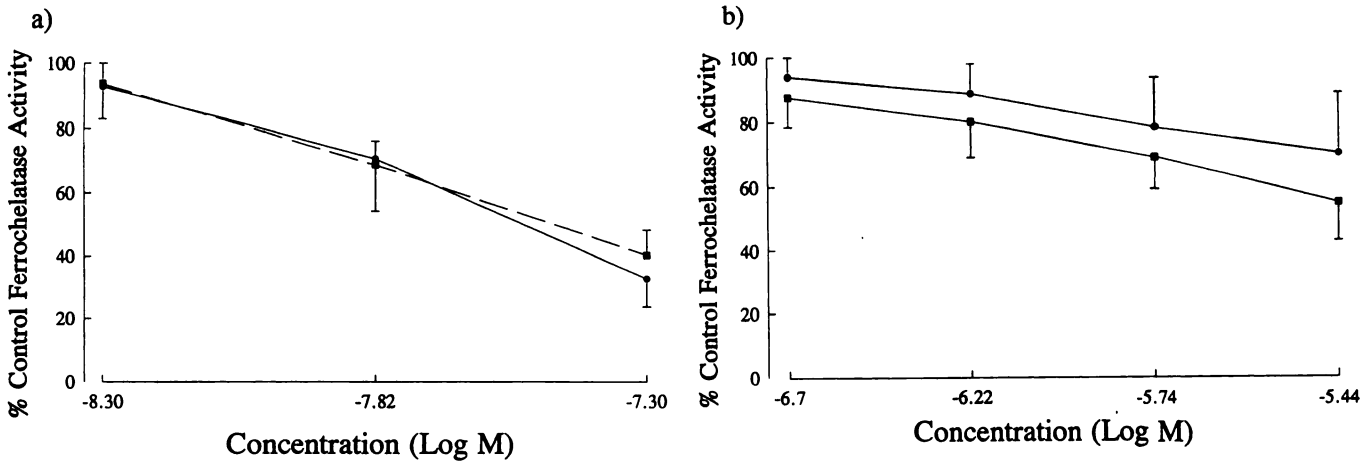

Fig. 5. Percentage of control ferrochelatase activity of chick embryo liver cell cultures after incubation with the N_A (●) or N_B (■) regioisomer of *N*-vinylPP (a) or the N_C (●) or N_D (■) regioisomer of *N*-vinylPP (b). Each point represents the mean ± standard deviation of four or five determinations. The 100% activity value of ferrochelatase expressed as mean ± standard deviation was as follows: N_A, 2.48 ± 0.32; N_B, 2.27 ± 0.25; N_C, 2.07 ± 0.11; and N_D, 2.07 ± 0.11 nmol mesoheme/mg protein/10 min.

TABLE 1

Comparison of the EC_{50} values for N_A and N_B regioisomers of biological *N*-ethylPP obtained from PB-pretreated rats after the administration of 4-ethyl DDC, biological *N*-vinylPP obtained from GLUT-pretreated chick embryos after the administration of TTMS, and synthetic *N*-ethylPP

All data are expressed as mean ± standard deviation of determinations from three or four experiments, except for the current synthetic *N*-ethylPP data, which were single determinations.

	EC_{50}	
	Previous data	Current data
	μM	
Biological N_A-ethylPP	0.009 ± 0.004[a]	
Biological N_B-ethylPP	0.050 ± 0.023[a]	
Biological N_A-vinylPP		0.030 ± 0.011
Biological N_B-vinylPP		0.035 ± 0.011
Synthetic N_A-ethylPP	0.014 ± 0.005[a]	0.019
Synthetic N_B-ethylPP	0.016 ± 0.006[a]	0.021

[a] Previous data from a paper published by our laboratory (10).

in the current study was within the same range of values as that measured previously (Table 1). It will be apparent (Table 1) that the potencies differ of the synthetic and biological regioisomers of *N*-ethylPP. The explanation for this difference is the following: In the chemical synthesis of *N*-ethylPP, alkylation occurs from both sides of the protoporphyrin IX ring, giving an optically inactive racemic mixture of regioisomers. In contrast, in rat hepatic P450, alkylation can occur only from one side of the protoporphyrin IX ring due to the orientation of the heme moiety within the active site, and an optically active *N*-ethylPP is formed (11). The 3.3-fold reduction in the potency of the N_A regioisomer of *N*-vinylPP relative to the N_A regioisomer of *N*-ethylPP suggest a reduction (~2.9 kJ/mol) in the binding energy of the N_A regioisomer of *N*-vinylPP to ferrochelatase. Due to limited crystallographic studies on the structure of *N*-vinylPPs, the origin of the difference can only be speculated. It is, however, known (35) that variations in the steric requirements of an *N*-alkyl substituent can alter the extent to which the pyrrolic rings of the porphyrin are canted. The differing steric demands of an ethyl group versus those of a vinyl group may alter the canting of the pyrrole rings, which, in turn, would affect the binding energy of the *N*-substituted pyrrole to ferrochelatase. Further structural information is required to clarify the origin of the difference in potency. Therefore, we were unable to obtain support for our hypothesis that the normal mechanism for ferrochelatase-catalyzed iron insertion has preference for an A—C ring tilt over a B—D ring tilt.

It is of interest that despite the larger amounts of *N*-alkylPP produced *in ovo* by TTMS (15.12 nmol/g wet weight liver) than by 4-ethyl DDC (0.81 nmol/g wet weight liver) (28), these two agents have comparable ferrochelatase-inhibitory activity (16). This is attributable to the increased amount and potency of the N_A regioisomer of *N*-ethylPP in the *N*-ethylPP mixture compared with the N_A regioisomer of *N*-vinylPP in the *N*-vinylPP mixture.

In conclusion, the novel findings from this study are that (i) a mixture of N_A, N_B, N_C, and N_D regioisomers of *N*-vinylPP previously demonstrated to accumulate in the livers of PB-pretreated rats after TTMS administration also accumulates in the livers of untreated and βNF-, DEX-, PB-, and GLUT-pretreated chick embryos; (ii) chick embryo hepatic P450 1A is not the source of *N*-vinylPP despite the fact that there is a 68% loss of P450 after TTMS administration to βNF-pretreated chick embryos; (iii) the N_B regioisomer constituted the lowest percentage of regioisomers (9–13%) and was present in the smallest quantity in untreated and drug-induced chick embryos (this observation supports the concept that ring B of heme is occluded by a protein residue or residues in the active site of P450); (iv) as was previously demonstrated with the regioisomers of *N*-ethylPP and other *N*-alkylPPs (26), the N_A and N_B regioisomers of *N*-vinylPP were markedly more potent inhibitors of ferrochelatase than the N_C and N_D regioisomers; and (v) the N_A regioisomer of *N*-vinylPP was found to be equipotent to the N_B regioisomer of *N*-vinylPP, a finding that did not support a previous proposal of an A—C pyrrole ring tilt in *N*-alkylPPs being important for ferrochelatase inhibition.

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