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Evidence for the Stereoselective Inhibition of Chick Embryo Hepatic Ferrochelatase by *N*-Alkylated Porphyrins

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SUMMARY

3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine and its 4-propyl analogue were administered to phenobarbital-pretreated rats. The *N*-alkylprotoporphyrins (N-alkylPPs) that were isolated from rat livers, *viz.*, *N*-ethylPP and *N*-propylPP, were found to have greater ferrochelatase-inhibitory potency than the corresponding synthetic *N*-alkylPPs. The *N*-ethylPP that was isolated from rat liver was found to contain 72% of the N_B plus N_A regioisomers, whereas synthetic *N*-ethylPP contained 40% of the N_B plus N_A regioisomers. In contrast, the *N*-propylPP that was isolated from rat liver contained the same amount of the $N_{B/A}$ regioisomer(s) as synthetic *N*-propylPP (33%). The N_B plus N_A regioisomers of *N*-ethylPP and the $N_{B/A}$ regioisomer(s) of *N*-propylPP that were isolated from rat liver were found to be significantly more potent than the corresponding synthetic regioisomers. We conclude that 1) the ferrochelatase-inhibitory potency of *N*-ethylPP that is isolated from rat liver is greater than that of synthetic *N*-ethylPP, due to differences in both regioisomer composition and the inhibitory potency of the N_B plus N_A regioisomers and stereoisomers, and 2) the ferrochelatase-inhibitory potency of *N*-propylPP that is isolated from rat liver is greater than that of synthetic *N*-propylPP, due solely to the difference in the ferrochelatase-inhibitory potency of the $N_{B/A}$ regioisomer(s) and stereoisomers. From the enhanced ferrochelatase-inhibitory potency of the N_B plus N_A regioisomers of *N*-ethylPP and the $N_{B/A}$ regioisomer(s) of *N*-propylPP that were isolated from rat liver, relative to the corresponding synthetic *N*-alkylPP regioisomers, it was inferred that 2- and 4-vinyl substituents located on pyrrole rings A and B contribute to the optimal binding of *N*-alkylPPs to the ferrochelatase active site.

The porphyrinogenic agent DDC (Fig. 1*a*), when administered to rodents, results in the inactivation of hepatic microsomal cytochrome P-450 and the hepatic accumulation of *N*-methylPP, a potent inhibitor of ferrochelatase (EC 4.99.1.1.) activity (1–4). DDC gives rise to *N*-methylPP as a consequence of its cytochrome P-450-mediated metabolism in the following way (5). One-electron oxidation of the dihydropyridine ring of DDC by cytochrome P-450 is thought to give rise to an unstable radical-cation intermediate that aromatizes both with retention of the 4-methyl group (approximately 99%) and with the elimination of the 4-methyl group (approximately 1%) as a free radical (6). This free radical, formed within the active site of cytochrome P-450, can alkylate any one of the four pyrrole nitrogens of cytochrome P-450 heme, leading to the formation of a mixture of N-methylPP regioisomers (Fig. 2*a*) and the mechanism-based inactivation of cytochrome P-450.

The ability of a 4-alkyl analogue of DDC to lower ferrochelatase activity is dependent on the formation of an *N*-alkylPP that can be accommodated readily within the ferrochelatase active site. The four regioisomers of *N*-methylPP (Fig. 2*a*), derived from DDC, are equally potent as inhibitors of ferrochelatase obtained from rat liver and chick embryo liver cell culture (7). In contrast, only the A (N_A) or B (N_B) pyrrole ring-substituted regioisomers of *N*-ethylPP (Fig. 2*b*) and *N*-propylPP (Fig. 2*c*), derived from 4-ethyl-DDC (Fig. 1*b*) and 4-propyl-DDC (Fig. 1*c*), respectively, are as potent as *N*-methylPP in inhibiting ferrochelatase; the C (N_C) and D (N_D) ring-substituted regioisomers are markedly less potent (8, 9). Recently, we have established that the ferrochelatase active site can accommodate *N*-alkylporphyrins with pyrrole ring substituents as large as hexyl or isobutyl on the A or B rings, but not on the C or D pyrrole rings (10).

We have shown that hepatic *N*-alkylPPs are formed in the chick embryo by isolating *N*-ethylPP from the livers of phenobarbital-pretreated chick embryos following the administration of 4-ethyl-DDC (11). This biologically derived *N*-ethylPP was found to be a more potent ferrochelatase inhibitor than synthetic *N*-ethylPP. We proposed two possible reasons for this observation. 1) The relative proportion of the regioisomers in the synthetic and biological preparations is different and the

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ABBREVIATIONS: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; PP, protoporphyrin IX; $N_{B/A}$ regioisomer(s) of *N*-alkylprotoporphyrin IX substituted on either the B or A or both rings; $N_{C/D}$, regioisomer(s) of *N*-alkylprotoporphyrin IX substituted on either the C or D or both rings.

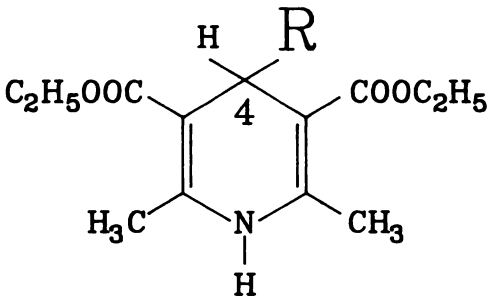

Fig. 1. *a*, *R* = —CH_3, DDC; *b*, *R* = —CH_2CH_3, 4-ethyl DDC; *c*, *R* = —$(CH_2)_2CH_3$, 4-propyl DDC.

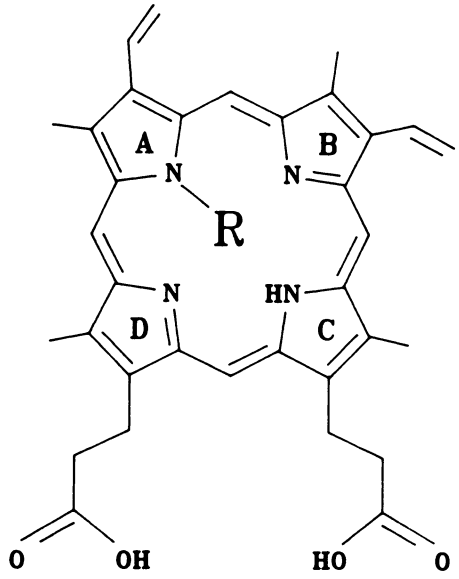

Fig. 2. *a*, *R* = methyl, the N_A regioisomer of *N*-methylPP; *b*, *R* = ethyl, the N_A regioisomer of *N*-ethylPP; *c*, *R* = propyl, the N_A regioisomer of *N*-propylPP. The other *N*-alkylPP regioisomers have the *N*-alkyl group located on the B (N_B), C (N_C), and D (N_D) pyrrole rings.

synthetic *N*-ethylPP contains less of the N_A and/or N_B regioisomers and more of the less potent N_C and/or N_D regioisomers than the biological *N*-ethylPP. 2) In the chemical synthesis of *N*-ethylPP, alkylation can occur from both sides of the PP ring, giving a racemic mixture for each of the regioisomers. In contrast, the orientation of the heme moiety within the active site of cytochrome P-450 is such that alkylation can occur from only one side of the PP ring; the resulting biologically derived *N*-alkylPP is consequently optically active. Ferrochelatase may be preferentially inhibited by the enantiomeric form of the *N*-alkylPP that is derived from the alkylation of the heme moiety of cytochrome P-450.

The objective of this study was to determine whether differences between the ferrochelatase inhibitory potency of biological and synthetic *N*-alkylPPs could be attributed to differences in regioisomeric or enantiomeric composition or to both factors. For this reason, *N*-ethylPP and *N*-propylPP were prepared both biologically and synthetically, separated into their regioisomers, and tested for ferrochelatase-inhibitory activity.

Materials and Methods

Source of compounds. DDC analogues were synthesized as described previously (5, 12, 13). The procedure for the synthesis of *N*-ethylPP and *N*-propylPP was essentially that of De Matteis *et al.* (14). The *N*-alkylPPs were purifed by two consecutive thin layer chromatographic systems, using Analtech 1000-μm silica gel G plates. The first system separated the *N*-alkylPP from the bulk of the unreacted PP dimethyl ester using a dichloromethane/methanol, 20:3 (v/v), solvent system. The *N*-alkylPPs, identified as green bands that fluoresced pink under UV light (R_f = 0.45–0.50), were scraped from the thin layer plates and eluted from the silica by repeated extractions with acetone. The acetone was subsequently removed using a rotary evaporator and the *N*-alkylPPs were dissolved in dichloromethane. Zinc acetate (50 μmol) in methanol (1.5 ml) was added to the dichloromethane solution in order to convert the *N*-alkylPPs to their corresponding zinc complexes (Zn-*N*-alkylPP). The Zn-*N*-alkylPPs were then purified by thin layer chromatography using a dichloromethane/acetone, 10:1 (v/v), solvent system. The Zn-*N*-alkylPPs (R_f = 0.75–0.8) were scraped from the plates, eluted from the silica with acetone, and evaporated to dryness. These Zn-*N*-alkylPPs will be referred to as synthetic Zn-*N*-ethylPP and synthetic Zn-*N*-propylPP. The synthetic samples were further purified and separated into regioisomers using high performance liquid chromatography, as described below for biologically derived *N*-alkylPPs.

Isolation, purification, and separation of *N*-alkylPP isomers. Groups of four to six male Sprague Dawley rats, weighing approximately 300 g, received sodium phenobarbital (80 mg/kg daily) dissolved in water (80 mg/ml), intraperitoneally, for 4 days. Each group received either 4-ethyl-DDC or 4-propyl-DDC (400 mg/kg) by the same route, in dimethyl sulfoxide (200 mg/ml), 36 hr after the final dose of sodium phenobarbital. Rats were decapitated 4–5 hr after dosing with the DDC analogue and the *N*-alkylPP dimethyl esters were isolated from the livers as previously described (10). The *N*-alkylPP dimethyl esters were converted to their zinc complexes and purified by two consecutive thin layer chromatographic systems, followed by high performance liquid chromatography (3, 4), as previously described (10).

The zinc cation was removed from the purified Zn-*N*-alkylPP dimethyl esters by treatment with 5% (v/v) H_2SO_4/methanol (15). Identification of *N*-alkylporphyrins was based on the electronic absorption spectra of their zinc complexes and zinc-free neutral dimethyl esters (3, 10, 12). The zinc-free neutral *N*-alkylPP dimethyl esters were separated into their constitutive regioisomers by high performance liquid chromatography (10). The identification of regioisomers was based on 1) their order of elution and 2) the electronic absorption spectra of their zinc complexes. The order of regioisomer elution that was observed previously (3, 4) with *N*-ethylPP and *N*-methylPP, using the same chromatographic system, was N_B, N_A, N_C, and N_D regioisomers. In previous studies, the zinc complexes of the N_A and N_B regioisomers, unequivocally identified by NMR spectroscopy, were found to have a shoulder on the long wavelength portion of their Soret bands (3). This shoulder was absent from the Soret band of the zinc complexes of the N_C and N_D regioisomers.

The *N*-alkylPPs that were isolated as described above from the livers of phenobarbital-pretreated rats will be referred to as biological *N*-ethylPP and biological *N*-propylPP.

Circular dichroism spectroscopy. The purified *N*-alkylPP regioisomers were converted to the corresponding chlorozinc complexes by washing the Zn-*N*-alkylPPs in dichloromethane with a 2% NaCl solution, as described previously (16). The *N*-alkylPPs were dissolved in spectroscopic grade dichloromethane (3.0 ml) to give a final concentration of 16.1 μM. Circular dichroism spectra were recorded between 350 and 550 nm, using an AVIV 60DS spectropolarimeter at 24.4° (1-cm cells; average time constant, 1.0 sec). The spectra were corrected by subtraction of the baseline observed with dichloromethane alone.

Ferrochelatase-inhibitory activity. The *N*-alkylPP dimethyl esters were hydrolyzed as previously described (10) and the *N*-alkylporphyrins were dissolved in 95% ethanol. A series of dilutions was prepared with 95% ethanol and 100-μl aliquots were transferred to the side arm of Thunberg tubes.

Details of the cell culture technique have been reported (17, 18). The chick embryo liver cells were maintained in 10-cm diameter plastic

dishes in 15 ml of serum-free Waymouth MD705/1 medium. The medium was replaced after an initial incubation period of 24 hr. After a further 24 hr, the medium was discarded and 5.0 ml of an ice-cold solution containing 0.25 M sucrose, 0.05 M Tris·HCl, and 1 mM EDTA at pH 8.2 was added to each dish. The cells were harvested with a rubber policeman, centrifuged (500 × *g*, 5 min), and washed with 1.0 ml/dish of the sucrose/Tris buffer. The cells were resuspended in 0.6 ml/dish of ice-cold 0.02 M Tris·HCl buffer (pH 8.2) and homogenized (Potter Elvehjem apparatus). Cell suspensions were then pooled and 0.9-ml aliquots were transferred to the side arm of the Thunberg tubes containing the *N*-alkylporphyrins dissolved in 95% ethanol (100 μl). The *N*-alkylporphyrins and ferrochelatase homogenates were incubated at room temperature for 20 min. The concentrations of *N*-alkylPP tested refer to their concentrations in the homogenate in the side arm of the Thunberg tube. After preincubation, the contents of the side arms were tipped into the reaction mixture in the main tube and incubated at 37° for 10 min, as described previously (18). The main tube contained mesoporphyrin IX (120 nmol), 1% (w/v) Tween 80 (0.3 ml), 0.2 mM Tris·HCl buffer, pH 8.2 (1.5 ml), 0.2 M dithioerythritol (60 μl), and 1.0 μM $FeSO_4$ (120 μl); the reaction was terminated by the addition of 0.4 M iodoacetamide (0.5 ml). Control ferrochelatase preparations were incubated with 95% ethanol (100 μl).

The amount of mesoheme formed was determined (10, 19) using the spectrum of the reduced minus oxidized pyridine mesohemochromogen, recorded from 580 to 520 nm on a Cary 219 spectrophotometer. Enzyme activity was calculated as nmol of mesoheme formed/mg of protein/10 min. Protein was measured by the Lowry protein assay (20).

Results

The yield of *N*-ethylPP and *N*-propylPP isolated from the livers of groups of four to six phenobarbital-pretreated rats (three experiments) following the administration of either 4-ethyl-DDC or 4-propyl-DDC was 41.0 ± 7.2 and 33.9 ± 8.8 nmol/rat, respectively. These determinations were made before high performance liquid chromatographic purification of the samples.

Regioisomer separation. The results of the high pressure liquid chromatographic separation of *N*-ethylPP and *N*-propylPP are shown in Figs. 3 and 4, respectively. *N*-EthylPP was separated into the expected four regioisomers with the order of regioisomer elution being N_B, N_A, N_C, and N_D (Fig. 3). The mixture of N_A and N_B regioisomers contained predominantly the N_A regioisomer (Fig. 3a). Characterization of the regioisomers is based on the order of elution previously established with synthetic *N*-methylPP and *N*-ethylPP, using the same chromatographic system (3, 4, 7, 8).

Representative chromatograms of the separation of the regioisomers of *N*-propylPP are shown in Fig. 4. The first peak, designated $N_{B/A}$ was identified as either the N_B or N_A or both regioisomers, on the basis of the retention time and the electronic absorption spectrum of the zinc-complexed dimethyl ester (i.e., shoulder on the Soret band). Similarly, the second peak, designated $N_{C/D}$ was identified as either the N_C or N_D or both regioisomer(s), on the basis of the retention time and the electronic absorption spectrum of the zinc-complexed dimethyl ester (i.e., no shoulder on the Soret band). No attempt was made to distinguish or separate the N_B from N_A or N_C from N_D regioisomers.

The regioisomer composition of the *N*-alkylPPs is expressed as a percentage of the total *N*-alkylPP recovered following regioisomer separation (Table 1) and quantitation by electronic absorption spectroscopy. As anticipated from Fig. 3, biological *N*-ethylPP contained significantly more of the N_B plus N_A regioisomers (72%) than synthetic material (37%). In contrast (Fig. 4), biological and synthetic *N*-propylPP contained a similar proportion of $N_{B/A}$ regioisomer(s) (i.e., 32.8% and 32.4%, respectively).

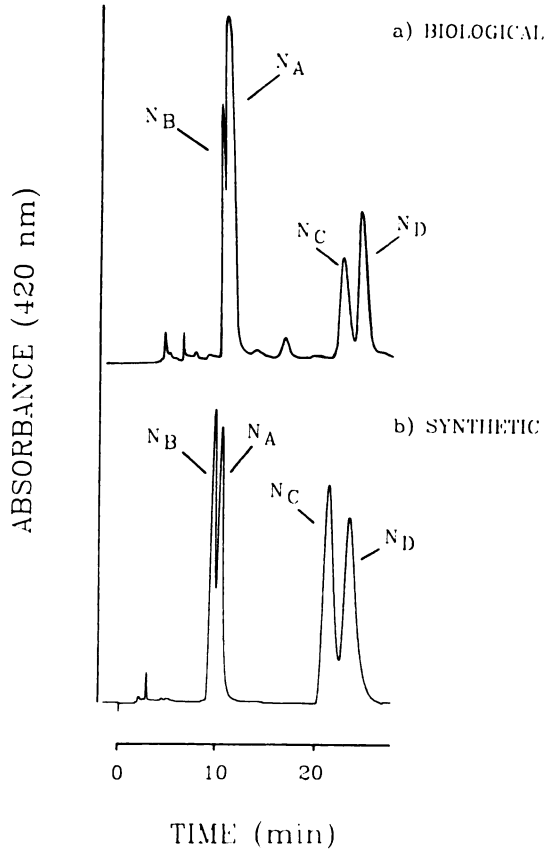

Fig. 3. High performance liquid chromatographic analysis of the biological (a) and synthetic (b) *N*-ethylPP dimethyl esters. A Partisil 10-PAC column (4.6 × 250 mm), eluted (1 ml/min) with hexane/tetrahydrofuran/methanol, 49.25:49.25:1.5 (v/v/v), and a variable wavelength detector set at 420 nm were used for the analysis.

Circular dichroism spectroscopy. The circular dichroism spectra of the N_B plus N_A regioisomers of *N*-ethylPP and the $N_{C/D}$ regioisomer(s) of *N*-propylPP are shown in Fig. 5. The N_B plus N_A regioisomers of biological *N*-ethylPP exhibited a prominent band of positive ellipticity in the 432 to 452 nm region (Fig. 5a) and the $N_{C/D}$ regioisomer(s) of *N*-propylPP exhibited a band of positive ellipticity in the 406 to 436 nm region (Fig. 5b). In contrast, circular dichroism spectra were not detected in samples of the N_B plus N_A and N_C plus N_D regioisomers of synthetic *N*-ethylPP and the $N_{B/A}$ and $N_{C/D}$ regioisomers of *N*-propylPP (results not shown).

Ferrochelatase-inhibitory activity. Using a crude ferrochelatase preparation obtained from chick embryo liver cell culture, the ferrochelatase-inhibitory activity of biological and synthetic *N*-alkylPPs were compared (Fig. 6). Biological *N*-ethylPP caused significantly greater ferrochelatase inhibition than synthetic *N*-ethylPP at concentrations of 0.04, 0.2, and 0.4 μM but not at 0.02 and 0.004 μM (Fig. 6a). At the highest concentrations tested, only the biological material (0.4 μM) was able to cause 100% inhibition of ferrochelatase activity; the synthetic material at the same concentration decreased ferrochelatase to approximately 36.2% of control activity. At twice

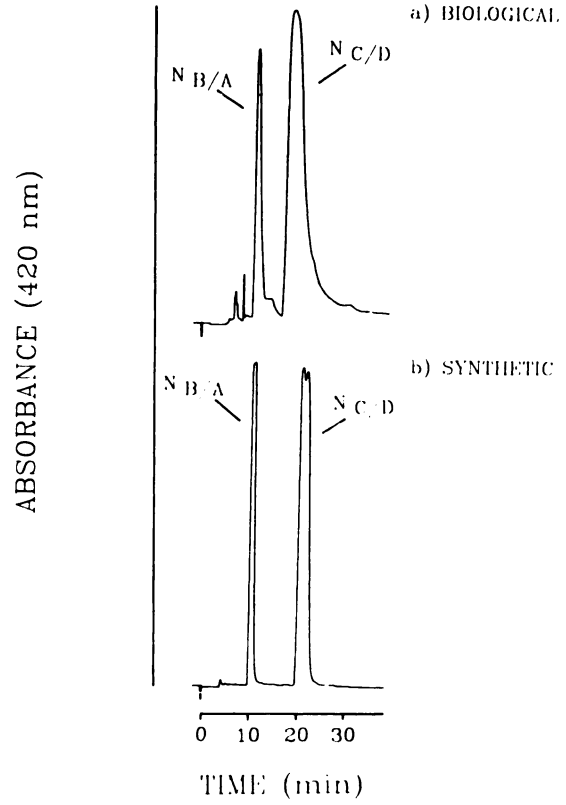

Fig. 4. High performance liquid chromatographic analysis of the biological (a) and synthetic (b) *N*-propylPP dimethyl esters. A Partisil 10-PAC column (4.6 × 250 mm), eluted (1 ml/min) with hexane/tetrahydrofuran/methanol, 49.25:49.25:1.5 (v/v/v), and a variable wavelength detector set at 420 nm were used for the analysis.

TABLE 1

Percentage of N_B and/or N_A regioisomers in the synthetic *N*-alkylPPs and *N*-alkylPPs isolated from phenobarbital-pretreated rats (biological) following administration of the corresponding 4-alkyl DDC analogue

Values are from three isolations or four to six replicates of synthetic material, presented as mean ± standard deviation.

	N_B and/or N_A regioisomers in *N*-alkylPP
	%
Synthetic *N*-ethylPP	39.6 ± 3.6
Biological *N*-ethylPP	72.0 ± 1.3
Synthetic *N*-propylPP	32.8 ± 1.6
Biological *N*-propylPP	32.4 ± 5.9

the concentration (0.8 μM), the synthetic *N*-ethylPP decreased ferrochelatase activity to 17.4% of control activity.

Biological *N*-propylPP caused greater inhibition of ferrochelatase activity than synthetic *N*-propylPP at 0.004, 0.02, and 0.04 μM but not at 0.2 and 0.4 μM (Fig. 6b). Ferrochelatase was completely inhibited by 0.4 μM levels of both the biological and synthetic material.

The ferrochelatase-inhibitory activity of the N_B plus N_A regioisomers of *N*-ethylPP and the $N_{B/A}$ regioisomers of *N*-propylPP was tested over a range of concentrations (0.004 to 0.4 μM) (Fig. 7, a and b, respectively). In both cases, the biological material caused significantly greater inhibition of ferrochelatase activity at concentrations of 0.004, 0.008, 0.02, and 0.04 μM. There was no significant difference in the ferrochelatase-inhibitory activity at the highest concentrations of 0.08 and 0.4 μM; 100% inhibition was attained at 0.4 μM with all N_A and/or N_B regioisomers.

The ferrochelatase-inhibitory activity of the biological and synthetic N_C plus N_D regioisomers of *N*-ethylPP and the biological and synthetic $N_{C/D}$ regioisomers of *N*-propylPP was also compared (results not shown). We were unable to show any consistent difference in ferrochelatase-inhibitory potency between the biological and synthetic material.

Discussion

We have used a chick embryo liver ferrochelatase preparation to compare the ferrochelatase-inhibitory activity of *N*-ethylPP and *N*-propylPP that were isolated from the livers of phenobarbital-pretreated rats with the ferrochelatase-inhibitory activity of synthetic *N*-ethylPP and *N*-propylPP. In previous studies, it has been shown that the four regioisomers of *N*-methylPP (7) and *N*-ethylPP (8, 10) interact with rat liver ferrochelatase in a manner comparable to that observed with chick embryo liver ferrochelatase. The biological *N*-alkylporphyrins were found to be more potent than their synthetic counterparts (Fig. 6). In order to explain these observations, we considered regioisomer composition. Biological *N*-ethylPP was found to consist of approximately twice as much of the N_B plus N_A regioisomers as the synthetic material (Table 1). Because the N_B plus N_A regioisomers of *N*-ethylPP are 30 to 100 times more potent than the N_C plus N_D regioisomers (8), the greater potency of the biological than of the synthetic *N*-ethylPP is explained, at least in part, by the regioisomer composition. In contrast, the regioisomer compositions of biological and synthetic *N*-propylPP were similar, with a ratio of $N_{B/A}$ to $N_{C/D}$ regioisomers of approximately 1:2 (Table 1). In this case, the difference in ferrochelatase-inhibitory potency between biological and synthetic *N*-propylPP cannot be explained on the basis of a difference in regioisomer composition.

In light of these observations, we compared the ferrochelatase-inhibitory potency of the purified N_B plus N_A regioisomers of biological and synthetic *N*-ethylPP and the ferrochelatase-inhibitory potency of the $N_{B/A}$ regioisomers of biological and synthetic *N*-propylPP. Circular dichroism spectra were not detectable in synthetic samples of the N_B plus N_A and N_C plus N_D regioisomers of *N*-ethylPP and the $N_{B/A}$ and $N_{C/D}$ regioisomers of *N*-propylPP (results not shown). This result is in accord with the expectation that, in the chemical synthesis of *N*-alkylPPs, alkylation can occur from both sides of the protoporphyrin ring, giving a racemic mixture for each of the regioisomers. The circular dichroism spectra of the N_B plus N_A regioisomers of *N*-ethylPP and the $N_{C/D}$ regioisomer(s) of *N*-propylPP showed that the biological *N*-alkylporphyrins are optically active (Fig. 5). These results are in agreement with previously published results (21). The biological N_B plus N_A regioisomers of *N*-ethylPP and the $N_{B/A}$ regioisomer(s) of *N*-propylPP were found to be significantly more potent than the corresponding synthetic regioisomers (Fig. 7). On the basis of these results we conclude that 1) the difference between the ferrochelatase-inhibitory potency of biological and synthetic *N*-ethylPP is due to differences in regioisomer composition and differences between the inhibitory potency of the biological and synthetic N_B plus N_A regioisomers and 2) the difference between the ferrochelatase-inhibitory potency of biological and synthetic N-propylPP is due solely to the difference between

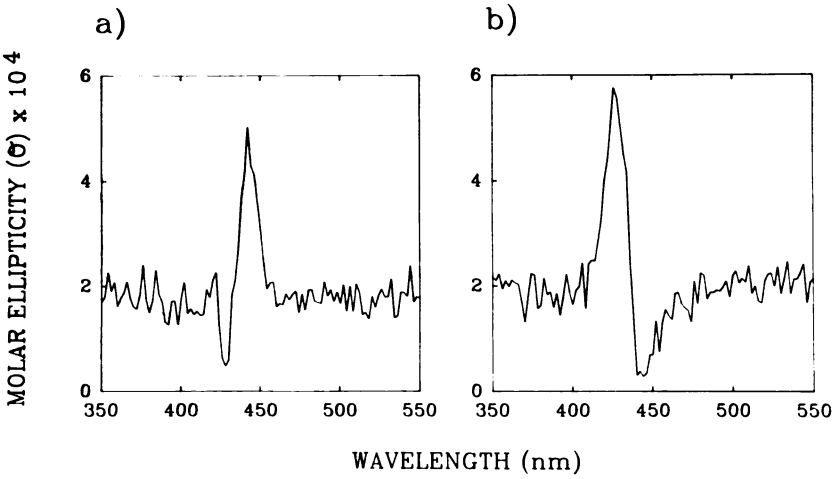

Fig. 5. Circular dichroism spectra of the N_B plus N_A regioisomers of *N*-ethylPP (a) and the $N_{C/D}$ regioisomer(s) of *N*-propylPP (b). Recordings were made at 2-nm intervals.

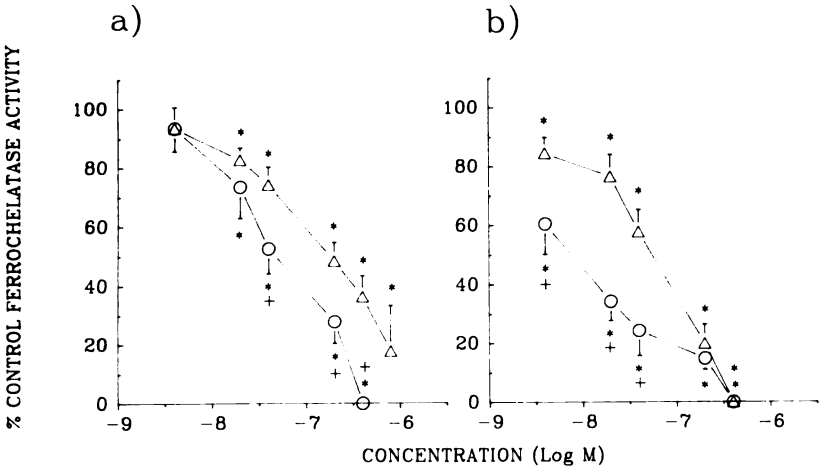

Fig. 6. Percentage of control ferrochelatase activity of chick embryo liver cell culture following incubation with biological (○) and synthetic (△) *N*-ethylPP (a) and *N*-propylPP (b). Control ferrochelatase activity was 3.06 ± 0.16 and 3.15 ± 0.45 nmol/mg of protein/10 min, respectively. *Significantly different from control; +significantly different from the same dose of synthetic *N*-alkylPP, as determined by a one-way analysis of variance ($p \leq 0.05$) and a Newman-Keuls test ($p \leq 0.05$).

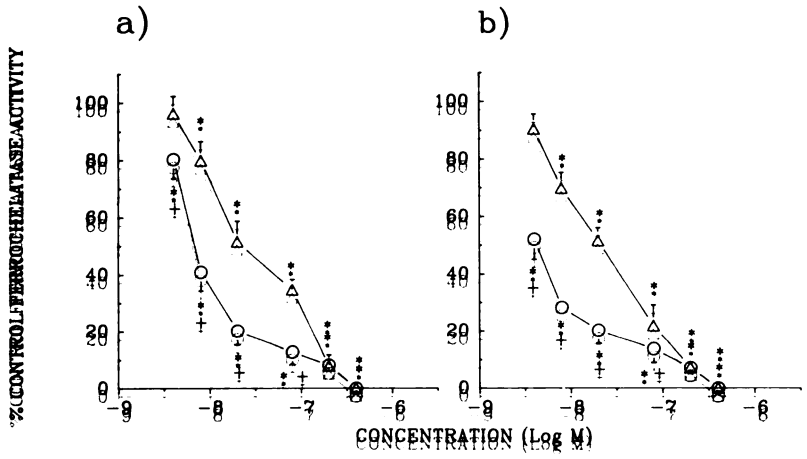

Fig. 7. Percentage of control ferrochelatase activity of chick embryo liver cell culture following incubation with the regioisomers of biological (○) and synthetic (△) *N*-ethylPP N_B plus N_A regioisomers (a) and *N*-propylPP $N_{B/A}$ regioisomer(s) (b). Control ferrochelatase activity was 3.46 ± 0.42 and 3.67 ± 0.22 nmol/mg of protein/10 min, respectively. *Significantly different from control; +significantly different from the same dose of synthetic *N*-alkylPP, as determined by a one-way analysis of variance ($p \leq 0.05$) and a Newman-Keuls test ($p \leq 0.05$).

the ferrochelatase-inhibitory potency of the synthetic and biological $N_{B/A}$ regioisomers.

The differences between the ferrochelatase-inhibitory potency of the regioisomers of the biological and synthetic *N*-ethylPP and *N*-propylPP indicate stereoselective binding of *N*-alkylPPs and may shed light on some aspects of the active site of ferrochelatase. With respect to its porphyrin substrate, ferrochelatase is a highly selective enzyme that utilizes only 6,7-dicarboxylic-substituted porphyrins (22). It has been proposed that the correct alignment of the PP macrocyle is due to ionic interaction between its two negatively charged propionic substituents and positively charged arginyl residues within the active site of the enzyme (23, 24). However, in addition to this ionic interaction, the vinyl groups located on the 2- and 4-positions of the PP are thought to interact with a hydrophobic portion of the enzyme (25). Replacement of the vinyl groups of PP with ethyl groups (mesoporphyrin IX) or hydrogen atoms (deuteroporphyrin IX) results in an increase in K_m (26). Protoporphyrin XIII, which resembles PP except that the vinyl groups are located on the 1- and 4-positions and not the 2- and 4-positions, also has a greater K_m, compared with porphyrin IX. Thus, it is clear that, for maximum affinity of the porphyrin

substrate for the active site of ferrochelatase, the type and location of substituent groups on the A and B pyrrole rings of the porphyrin is also an important consideration. Our studies on the inhibition of ferrochelatase by both synthetic and biological N_A plus N_B regioisomers of *N*-ethylPP reinforce this conclusion, as described below.

From the circular dichroism studies of Ortiz de Montellano *et al.* (21), it can be deduced that the A ring regioisomer of the biological *N*-ethylPP, obtained from rat liver, has predominantly if not exclusively the absolute configuration shown in Fig. 8a; this is based upon the observation that the N_A regioisomer is the predominant one by high performance liquid chromatography (Fig. 3a) and that the circular dichroism spectrum displayed positive ellipticity (Fig. 5a). It is presumed that the biological N_B-, N_C-, and N_D-ethylPPs have the same absolute configuration, i.e., are formed by alkylation on the same face of the cytochrome P-450 heme. Consequently, the biological N_A and N_B, regioisomers consist predominantly of the two stereoisomers shown in Fig. 8, a and c, whereas the synthetic mixture of A and B ring regioisomers consists of two pairs of enantiomers, *viz.*, N_A-ethyl (Fig. 8a) and epi-N_A-ethyl (Fig. 8b) and N_B-ethyl (Fig. 8c) and epi-N_B-ethyl (Fig. 8d). The term epi is used to denote the opposite stereochemistry at the chiral center. It should be noted that, by rotation from Fig. 8b to Fig. 8e, the epi-N_A regioisomer is an "analogue" of the N_B regioisomer (Fig. 8c), differing only in the position of the vinyl and methyl groups on the A and B rings. In a similar way, the epi-N_B regioisomer (Fig. 8f) is an "analogue" of the N_A regioisomer (Fig. 8a).

We have previously shown (8) that the synthetic N_A regioisomer of *N*-ethylPP (a racemic mixture of N_A and epi-N_A stereoisomers; Fig. 8) is equipotent with the synthetic N_B regioisomer (a racemic mixture of N_B and epi-N_B stereoisomers; Fig. 8). In the present study, the mixture of A and B ring regioisomers obtained from rat liver contains predominantly the N_A stereoisomer (Fig. 8a) and the N_B stereoisomer (Fig. 8c). This biologically derived mixture is *more potent* at inhibiting ferrochelatase than the synthetic mixture. Consequently, the four isomers shown in Fig. 8 cannot be of equal potency.

One way in which this result can be interpreted is based upon the assumption that the N_A regioisomer (Fig. 8a) is equipotent with the N_B regioisomer (Fig. 8c). It follows that the epi-N_A regioisomer (Fig. 8e), an analogue of the N_B regioisomer, is less potent than the N_B regioisomer and the epi-N_B regioisomer (Fig. 8f), an analogue of the N_A regioisomer, is less potent than the N_A regioisomer. The higher potency of the N_A and N_B regioisomers, as compared with their enantiomers, suggests that the ferrochelatase active site must be discriminating among these two pairs of enantiomers by virtue of the difference in position of the methyl and vinyl groups. The interaction of the methyl and vinyl substituents with the active site of ferrochelatase should be considered a hydrophobic interaction, with a possible steric preference for the positioning of the methyl and vinyl groups. In order to confirm this interpretation, it will be necessary to prove that the N_A-ethylPP regioisomer (Fig. 8a) is equipotent to the N_B-ethylPP regioisomer (Fig. 8c).

In summary, the difference in the ferrochelatase-inhibitory potency of biological and synthetic *N*-ethylPP is due to both a difference in the regioisomer composition and a difference in the ferrochelatase-inhibitory potency of the N_B plus N_A regioisomers and stereoisomers. On the other hand, a difference in the ferrochelatase-inhibitory potency of biological and synthetic *N*-propylPP is due solely to the difference in the ferrochelatase-inhibitory activity of the $N_{B/A}$ regioisomer(s) and stereoisomers. From the enhanced ferrochelatase-inhibitory potency of the N_B plus N_A regioisomers of *N*-ethylPP that are

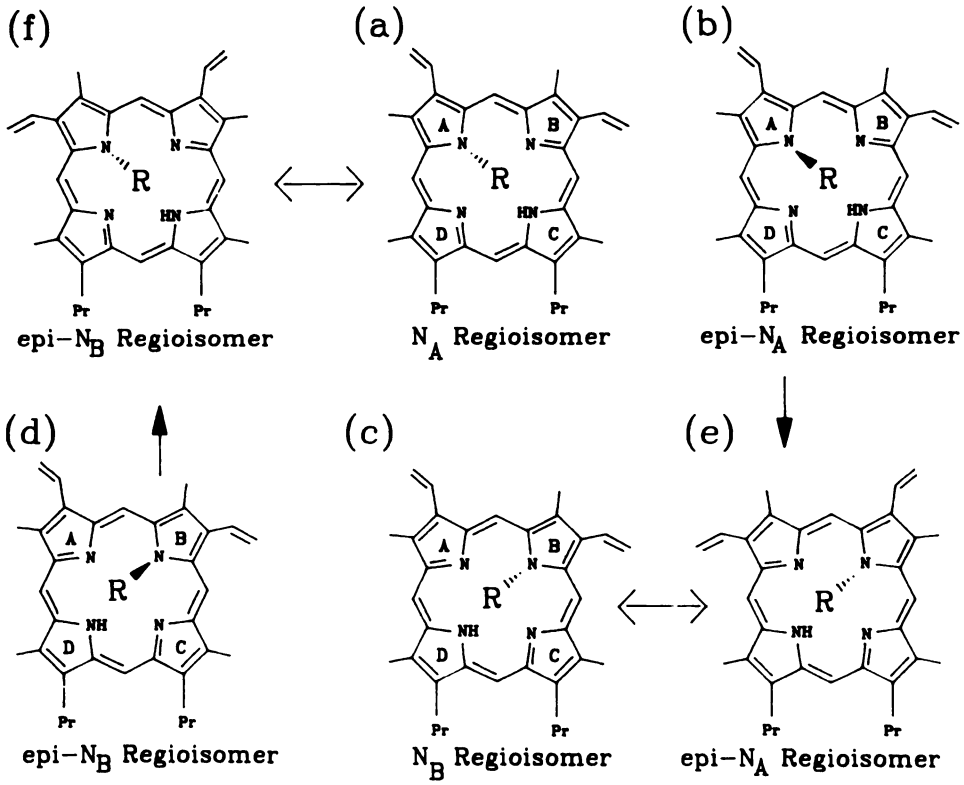

Fig. 8. Configuration of the N_A and N_B regioisomers of *N*-ethylPP (*R* = ethyl).

isolated from rat liver, relative to the synthetic N_B plus N_A regioisomers, it is inferred that the 2- and 4-vinyl substituents located on pyrrole rings A and B contribute to the optimal binding of *N*-alkylPPs to a hydrophobic region of the ferrochelatase active site.

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