0026-895X/88/01080-07$02.00/0

MOLECULAR PHARMACOLOGY, 34:80–86

Differential Inhibition of Hepatic Ferrochelatase By Regioisomers of *N*-Butyl-, *N*-Pentyl-, *N*-Hexyl-, and *N*-Isobutylprotoporphyrin IX

S. A. MCCLUSKEY, G. S. MARKS, R. A. WHITNEY, and P. R. ORTIZ DE MONTELLANO

Department of Pharmacology and Toxicology (S.A.M., G.S.M.) and Chemistry (R.A.W.), Queen's University, Kingston, Ontario K7L 3N6, and Department of Pharmaceutical Chemistry, School of Pharmacy and Liver Center, University of California, San Francisco, California 94143 (P.R.O.M.)

Received March 4, 1988; Accepted April 27, 1988

SUMMARY

A series of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), *viz.* 4-butyl DDC, 4-pentyl DDC, and 4-hexyl DDC was administered to phenobarbital-pretreated rats. The *N*-alkylprotoporphyrins (*N*-alkylPP) isolated from the rat livers were separated into regioisomers by means of high performance liquid chromatography; the N_B or N_A ($N_{B/A}$) regioisomers constituted 19–26% of the total regioisomers. Considerable ferrochelatase-inhibitory activity was found in the $N_{B/A}$ regioisomers; the N_C or N_D ($N_{C/D}$) regioisomers had little ferrochelatase-inhibitory activity. These findings supported the idea that the ferrochelatase active site could accommodate alkyl groups larger than methyl only if they were present on the nitrogens of the A or B pyrrole rings of the *N*-alkylPP. The inactivity of 4-isobutyl DDC as a ferrochelatase-lowering agent was investigated. After injection of 4-isobutyl-DDC into phenobarbital-pretreated rats, *N*-isobutylPP was isolated from the rat livers and separated into its regioisomers; the $N_{B/A}$ regioisomer constituted 3.8% of the total regioisomers. The $N_{B/A}$ regioisomer was found to have appreciable ferrochelatase-inhibitory activity whereas the $N_{C/D}$ regioisomer was inactive. The inactivity of 4-isobutyl-DDC as a ferrochelatase-lowering agent was attributed to the small amount of $N_{B/A}$ regioisomer present in the *N*-isobutylPP regioisomer mixture.

DDC (Fig. 1*a*) when administered to rodents results in the hepatic accumulation of *N*-methylPP, a potent inhibitor of ferrochelatase (EC 4.99.1.1) activity (1–4). The four regioisomers of *N*-methylPP (Fig. 2*a*) are equally potent as inhibitors of ferrochelatase from rat liver and from cultured chick embryo liver cells (5). The administration of 4-ethyl-DDC (Fig. 1*b*) to rodents (4) or chick embryos (6) results in the hepatic accumulation of *N*-ethylPP (Fig. 2*b*). The N_A and N_B regioisomers of *N*-ethylPP inhibit ferrochelatase as effectively as the four regioisomers of *N*-methylPP whereas the N_C and N_D regioisomers of *N*-ethylPP are 30 to 100 times less potent (7). The steric constraints on the *N*-alkyl group are therefore more marked when the group is located on the propionic acid-substituted C and D pyrrole rings than on the vinyl-substituted A and B pyrrole rings. Because the ferrochelatase protoporphyrin-binding site does not appear to discriminate between a methyl and an ethyl group in the A and B ring-substituted isomers, it appeared possible that even larger alkyl substituents would be compatible with inhibitory activity. This idea was supported by the ability of 4-propyl-DDC (Fig. 1*c*) to lower ferrochelatase activity in the chick embryo liver cell culture (8) and the ability of the N_A and N_B regioisomers of *N*-propylPP to inhibit ferrochelatase activity (9); the N_C and N_D regioisomers displayed little activity.

We have shown that the 4-butyl, 4-pentyl, and 4-hexyl analogues of DDC (Fig. 1, *d*, *e*, and *f*) have ferrochelatase-lowering activity in chick embryo liver cell culture (10). The ferrochelatase-lowering activity of these DDC analogues was attributed to the formation of *N*-alkylporphyrins in cell culture. This inference was supported by the isolation of *N*-butylPP, *N*-pentylPP, and *N*-hexylPP from the livers of phenobarbital-pretreated rats after the administration of the corresponding 4-alkyl DDC analogue. In contrast, 4-isobutyl DDC (Fig. 1*g*) did not exhibit ferrochelatase-lowering activity, despite its ability to cause the hepatic accumulation of *N*-isobutylPP in phenobarbital-pretreated rats.

If our assumption that the ferrochelatase active site can accommodate larger alkyl groups on only the A and B pyrrole rings is correct, then the ferrochelatase-lowering activity of the 4-butyl-, 4-pentyl-, and 4-hexyl-DDC must be due to the formation of the N_A and/or the N_B regioisomers of the corresponding *N*-alkylporphyrin. In addition, the inability of 4-isobutyl-DDC to lower ferrochelatase activity must be due to either 1)

This work was supported by the Medical Research Council of Canada and National Institutes of Health Grant GM 25515.

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

exclusive or nearly exclusive formation of the N_C and/or N_D regioisomers of *N*-isobutylPP or 2) the fact that a branched alkyl group is sterically incompatible with the ferrochelatase active site even when located on pyrrole rings A or B. To resolve these questions the *N*-alkylporphyrins derived from the administration of 4-butyl-, 4-pentyl-, 4-hexyl-, and 4-isobutyl-DDC analogues to rats were isolated and separated into their constitutive regioisomers. The ferrochelatase-inhibitory activity of the *N*-alkylPP regioisomers was tested using a ferrochelatase preparation obtained from chick embryo liver cell culture.

Experimental Procedures

Source of compounds. DDC analogues were synthesized as described previously (10–12). The procedure for the synthesis of *N*-ethylPP was essentially that of De Matteis *et al.* (13).

Isolation, purification, and separation of *N*-alkylPP isomers. Groups of four 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-butyl-DDC, 4-pentyl-DDC, 4-hexyl-DDC, or 4-isobutyl-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 their livers were perfused *in situ* with ice-cold 1.15% potassium chloride solution. Livers were homogenized in a Waring blender with ice-cold 5% (v/v) H_2SO_4/methanol (150 ml/liver) and the mixture was stored in the dark at 4° for 17–20 hr. The mixture was then centrifuged (500 × *g*, 5 min) and the 5% H_2SO_4/methanol supernatant was collected. Pellets were washed with methanol (50 ml/liver) and the methanol washing was added to the 5% H_2SO_4/methanol supernatant. The methanol solution was then diluted with an equal volume of water and extracted twice with dichloromethane. The dichloromethane solution was washed once with 5% sodium bicarbonate solution and twice with water and dried over anhydrous sodium sulfate. The dichloromethane solution was filtered, then evaporated to approximately 20 ml, and zinc acetate (25 μmol) in methanol —(1 ml) was added; the mixture was evaporated to dryness. The zinc-complexed *N*-alkylporphyrins were then purified by two consecutive thin layer chromatographic systems using Analtech 2000-μm and 500-μm silica gel G plates developed with dichloromethane/acetone, 4:1 (v/v), and chloroform/acetone, 10:1 (v/v), respectively. Green bands that fluoresced red under long wavelength (ultraviolet) light were eluted from the silica with acetone as described previously (10). The Zn *N*-alkylporphyrin dimethyl esters were further purified by high performance liquid chromatography (3, 4) on a Partisil-10 PAC column (4.6 × 250 mm) eluted using a 30-min linear gradient (0–100%) of methanol into hexane/tetrahydrofuran, 1:1 (v/v).

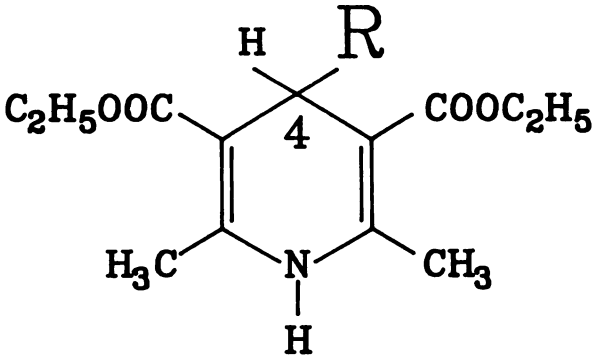

Fig. 1. Structure of dihydropyridine analogues. *a*, $R = -CH_3$, DDC; b, $R = -CH_2CH_3$, 4-ethyl-DDC; *c*, $R = -(CH_2)_2CH_3$, 4-propyl-DDC; *d*, $R = -(CH_2)_3CH_3$, 4-butyl-DDC; *e*, $R = -(CH_2)_4CH_3$, 4-pentyl-DDC; *f*, $R = -(CH_2)_5CH_3$, 4-hexyl-DDC; *g*, $R = -CH_2CH(CH_3)_2$, 4-isobutyl-DDC.

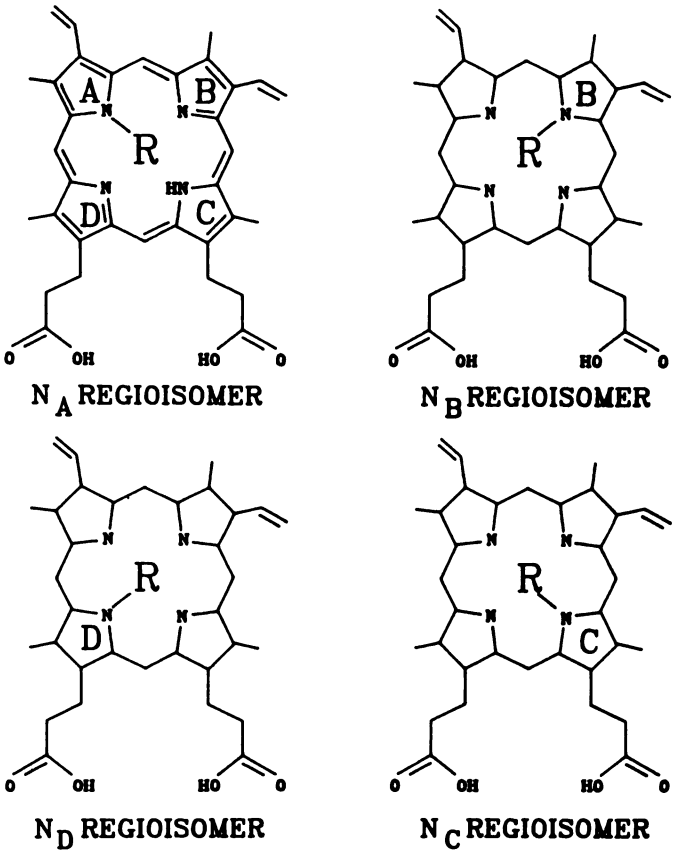

Fig. 2. *a*, *R* = methyl, the four possible regioisomers of *N*-methylPP; *b*, *R* = ethyl, the four possible regioisomers of *N*-ethylPP.

The zinc cation was removed from the purified Zn *N*-alkylporphyrin dimethyl esters by treatment with 5% (v/v) H_2SO_4/methanol (14). Identification of *N*-alkylporphyrins was based on the electronic absorption spectra of their zinc complexes and zinc-free neutral dimethyl esters. In addition, the field desorption mass spectra of *N*-alkylPP were obtained by the Mass Spectrometry Resource, School of Pharmacy, University of California.

The zinc-free neutral *N*-alkylPP dimethyl esters were separated into their constitutive regioisomers by high performance liquid chromatography (3, 4) using a Partisil-10 PAC column (4.6 × 250 mm) eluted isocratically with hexane/tetrahydrofuran/methanol 48.5:48.5:3.0 (v/v/v). 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 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. It was anticipated that this order of elution would be observed with other *N*-alkylPP isomers.

In previous studies, the zinc complexes of the N_A and N_B regioisomers 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. This property has been used to differentiate the N_A or N_B from the N_C or N_D regioisomers. Quantitation of *N*-alkylporphyrins in dichloromethane was accomplished using the molar absorbance values of 127,800 at 420 nm and 124,000 at 432 nm for the zinc-free neutral and zinc-complexed dimethyl esters, respectively (3). All procedures were carried out in subdued light.

Ferrochelatase-inhibitory activity. The *N*-alkylPP dimethyl esters were hydrolyzed by treatment with 6.0 N HCL overnight at room temperature in the dark (14). After removal of 6.0 N HCl under a stream of nitrogen, 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 sidearm of Thunberg tubes.

Details of the cell culture technique have been reported (15, 16). The chick embryo liver cells were maintained in 10-cm diameter plastic dishes in 15 ml of serum-free Waymouth MD705/1 medium supplemented with penicillin G sodium, streptomycin sulfate, insulin, and L-thyroxine sodium pentahydrate. The medium was replaced after an initial incubation period of 24 hr. After an additional 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 sucrose/Tris buffer. The cells were resuspended in 0.6 ml/dish 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 sidearm 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. After preincubation, the contents of the sidearms were then tipped into the reaction mixture in the main tube and incubated at 37° for 10 min as described previously (16). 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 using the spectrum of the reduced minus oxidized pyridine mesohemochromogen recorded from 580 to 520 nm on a Cary 219 spectrophotometer. The amount of mesoheme formed was calculated using the equation $\Delta\epsilon_{mM} = (\epsilon_{mM}^{547nm} - \epsilon_{nM}^{531nm}) = 21.7$ (17). Background $\Delta\epsilon$ was determined using control samples in which iodoacetamide had been added to the reaction mixture before tipping the ferrochelatase preparation from the sidearm of the Thunberg tube into the main tube; this value was subtracted from all sample $\Delta\epsilon$ values. Samples having $\Delta\epsilon$ values <1.5 × background $\Delta\epsilon$ were considered as having insignificant ferrochelatase activity (i.e., zero mesoheme formed). Enzyme activity was expressed as nmol of mesoheme formed/mg of protein/10 min. Protein was measured by the method of Lowry *et al.* (18).

Results

After purification of the *N*-alkylporphyrins by two thin layer chromatographic procedures, the amount of *N*-alkylPP isolated per phenobarbital-pretreated rat (± SD, $n = 4$–6) was as follows: *N*-butylPP, 13.5 ± 6.5 μg; *N*-isobutylPP, 13.6 ± 6.6 μg; *N*-pentylPP, 30.5 ± 15.7 μg; and *N*-hexylPP, 26.5 ± 18 μg.

N-Alkylporphyrins isolated from the livers of phenobarbital-pretreated rats were identified by the electronic absorption spectra of their zinc-free neutral and zinc-complexed dimethyl esters as described previously (10). The electronic absorption spectra of *N*-isobutylPP, which has not been reported previously, was very similar to the spectra of other zinc-free neutral and zinc-complexed *N*-alkylporphyrins (Table 1).

The field desorption mass spectra of the porphyrins obtained with 4-pentyl, 4-hexyl, and 4-isobutyl analogues of DDC provide further evidence for their structure. The monoprotonated molecular ion of these porphyrins (*m/z* 661, *m/z*, 675, and *m/z* 647, respectively) are those expected for structures constructed from PP dimethyl ester and the pentyl, hexyl, and isobutyl moiety. The mass spectra for *N*-pentylPP and *N*-hexylPP were reported previously (10). The results for the *N*-isobutylPP were obtained in the present study.

Regioisomer separation. The results of the high performance liquid chromatographic separation of the expected four regioisomers of *N*-ethylPP dimethyl ester are shown in Fig. 3. The N_B, N_A, N_C, and N_D order of regioisomer elution is based on the previously published chromatographic separation of synthetic *N*-methylPP and *N*-ethylPP using the same chromatographic system (3, 4, 5, 7). Samples of the N_B plus N_A and the N_C plus N_D regioisomers were collected and shown to have the expected electronic absorption spectra. The electronic absorption spectra of the zinc complexes of the N_B plus N_A and N_C plus N_D regioisomers prepared by the addition of zinc acetate in methanol are shown in Fig. 4. The N_B plus N_A regioisomers can be distinguished from the N_C plus N_D regioisomers by a shoulder that appears on the Soret band (432 nm) of the N_B plus N_A regioisomers.

TABLE 1

Electronic absorption spectra in dichloromethane of the zinc complexes and the zinc-free neutral dimethyl esters of *N*-ethylPP (synthetic), N-butylPP, and N-isobutylPP

	Absorption Maxima							
	Zinc Complex				Zinc-Free Neutral			
	Soret	β	α	α^1	Soret	IV	III	II
	nm							
N-EthylPP	432	546	592	632	420	512	544	594
N-IsobutylPP	432	544	594	632	418	512	546	592
N-ButylPP	432	544	592	632	420	514	544	592

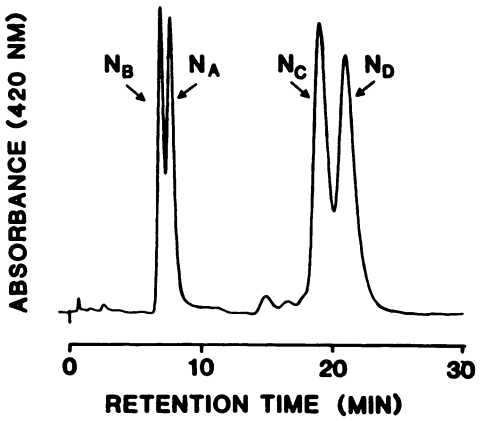

Fig. 3. High performance liquid chromatography analysis of the synthetic *N*-ethylPP dimethyl esters. A Partisil-10-PAC column (4.6 × 250 mm), eluted (1 ml/min) with hexane/tetrahydrofuran/methanol, 48.5:48.5:3.0 (v/v/v), and a variable wavelength detector, set at 420 nm, were used for the analysis.

Representative chromatograms of the separation of the regioisomers of *N*-butyl-, *N*-pentyl-, *N*-hexyl-, and *N*-isobutylPP are shown in Fig. 5, a, b, c, and d, respectively. In each case the first peak was identified as either the N_B or N_A regioisomer on the basis of the retention time (7–10 min) and electronic absorption spectrum of the zinc-complexed dimethyl ester. Similarly, the second peak (retention time, 17–22 min) was identified as either the N_C or N_D regioisomer. No attempt was made to distinguish the N_A from N_B or the N_C from N_D regioisomers. For this reason regioisomers are described as $N_{B/A}$ and $N_{C/D}$ to indicate that the *N*-alkylporphyrin is either the N_A or N_B and N_C or N_D regioisomers, respectively.

The composition of the *N*-alkylporphyrins isolated from phenobarbital-pretreated rats after the administration of their respective 4-alkyl-DDC analogues is expressed as a percentage of the total *N*-alkylporphyrin recovered after isomer separation (Table 2). The major fraction of *N*-alkylporphyrin in each case was the $N_{C/D}$ regioisomer. The percentage of $N_{B/A}$ in *N*-butyl-, *N*-pentyl-, and *N*-hexylPP regioisomers varied from 19 to 26%.

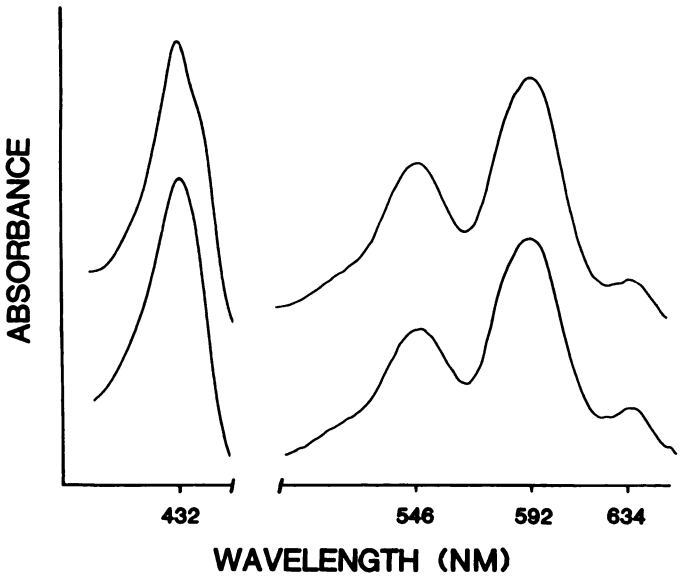

Fig. 4. Electronic absorption spectra of the zinc complexes of the N_B plus N_A (*top*) and N_C plus N_D (*bottom*) regioisomers of synthetic *N*-ethylPP dimethyl ester. The Soret band was recorded at a 10-fold higher attenuation of the recorder.

In contrast, the percentage of $N_{B/A}$ in the mixture of *N*-isobutylPP regioisomers was approximately 3.8%.

Ferrochelatase-inhibitory activity. Using a crude ferrochelatase preparation obtained from chick embryo liver cell culture, the ferrochelatase-inhibitory activity of the regioisomers of *N*-butylPP, *N*-pentylPP, *N*-hexylPP, and *N*-isobutylPP was compared with the ferrochelatase-inhibitory activity of the regioisomers of synthetic *N*-ethylPP (Fig. 6, a, b, c, and d respectively). The $N_{B/A}$ regioisomer of *N*-butylPP, *N*-pentylPP, *N*-hexylPP, and *N*-isobutylPP caused a significant inhibition of ferrochelatase activity at 340 and 34 pmol/ml (but not at 3.4 pmol/ml) but were less potent than the N_B plus N_A regioisomers of *N*-ethylPP. It is noteworthy that complete inhibition of ferrochelatase activity was obtained with the N_B plus N_A regioisomers of *N*-ethylPP (340 pmol/ml) but not with the same dose of the $N_{B/A}$ regioisomers of *N*-butylPP, *N*-pentylPP, *N*-hexylPP, or *N*-isobutylPP. Because of the limited amount of the $N_{B/A}$ regioisomer of the latter *N*-alkylPPs, it was not possible to determine whether complete inhibition of ferrochelatase was achievable at higher concentrations and thus whether these compounds had the same efficacy as *N*-ethylPP.

The $N_{C/D}$ regioisomer of *N*-isobutylPP did not cause significant inhibition of ferrochelatase activity at the concentrations tested. The $N_{C/D}$ regioisomers of the *N*-butylPP, *N*-pentylPP, and *N*-hexylPP were found to produce a significant inhibition of ferrochelatase activity at a concentration of 340 pmol/ml, but not at the lower concentrations tested. The ferrochelatase-inhibitory activity of the $N_{C/D}$ regioisomers of the *N*-alkylporphyrins isolated from rat livers was not significantly different from the ferrochelatase-inhibitory activity of the N_C plus N_D regioisomers of synthetic *N*-ethylPP.

Discussion

The first objective of this study was to ascertain whether the ferrochelatase-inhibitory activity of *N*-butylPP, *N*-pentylPP, and *N*-hexylPP could be accounted for by the presence of N_A and/or N_B regioisomers. Considerable ferrochelatase-inhibitory activity was found in the $N_{B/A}$ regioisomers; the $N_{C/D}$ regioisomers had little ferrochelatase-inhibitory activity (Fig. 6, a, b, and c). On the basis of the above observations, we conclude that the ferrochelatase-lowering activity of 4-butyl-DDC, 4-pentyl-DDC, and 4-hexyl-DDC is due to the formation of the $N_{B/A}$ regioisomer of the corresponding *N*-alkylporphyrin. The second objective was to ascertain the reason for the inability of 4-isobutyl-DDC to lower ferrochelatase activity (10). The $N_{C/D}$ regioisomer of *N*-isobutylPP did not produce significant inhibition of ferrochelatase activity at any of the concentrations tested. The purified $N_{B/A}$ regioisomer was found to inhibit ferrochelatase with a potency similar to that of the other *N*-alkylPP $N_{B/A}$ regioisomers studied (e.g., *N*-hexylPP; Fig. 6). The inability of 4-isobutyl-DDC to lower ferrochelatase activity cannot be explained by the fact that a branched chain alkyl group located on either the A or B pyrrole rings is sterically incompatible with the ferrochelatase active site. The $N_{B/A}$ regioisomers constituted only 3.8% of the *N*-isobutylPP mixture. This compares with 19–26% $N_{B/A}$ regioisomers found in the regioisomer mixtures of *N*-butylPP, *N*-pentylPP, and *N*-hexylPP. The inactivity of 4-isobutyl DDC as a ferrochelatase-lowering agent is therefore attributable, at least in part, to the nearly exclusive formation of the N_C and/or N_D regioisomers of *N*-isobutylPP.

An additional factor involved in the failure to demonstrate ferrochelatase-lowering activity when 4-isobutyl DDC was added to chick embryo hepatocyte culture may be the following: the composition of cytochrome P-450 isozymes in the hepatocyte culture would be expected to differ from that in the rat liver. Because the formation of *N*-alkylPP is likely to be dependent upon interaction of 4-isobutyl DDC with specific isozymes of cytochrome P-450 (11, 19), it is possible that the specific isozymes of cytochrome P-450 required for the formation of the $N_{B/A}$ regioisomers of *N*-isobutylPP in rat liver were absent or present at very low levels in chick embryo hepatocyte culture.

In a previous study (20) we compared the ferrochelatase-lowering activity of the DDC analogues (FIg. 1, *a–g*) in chick embryo hepatocyte culture. Ferrochelatase-lowering activity decreased as the length and bulk of the 4-alkyl chain increased. Thus 4-pentyl DDC was less active than DDC, 4-ethyl DDC, and 4-propyl DDC but more active than 4-hexyl DDC; 4-isobutyl DDC was inactive. The results obtained in the present study and by other investigators on the regioisomer composition of *N*-alkylporphyrins helps to shed light on the differences in ferrochelatase-inhibitory activity observed with DDC analogues. When DDC was administered to phenobarbital-pretreated rats, the ratio of N_B plus N_A to N_C plus N_D regioisomers in *N*-methylPP isolated from rat livers was 6.5:1 (3). The administration of 4-ethyl DDC to phenobarbital-pretreated rats resulted in the accumulation in the rat livers of an *N*-ethylPP with a ratio of N_B plus N_A to N_C plus N_D regioisomers of 2:1 (19). In the present study a ratio of $N_{B/A}$ to $N_{C/D}$ regioisomers of between 1:4 and 1:3 was observed with *N*-butylPP, *N*-pentylPP, and *N*-hexylPP and 1:25 with *N*-isobutylPP. It thus appears that the bulkier the 4-alkyl substituent of DDC, the lower the ratio of $N_{B/A}$ to $N_{C/D}$ regioisomers formed. The $N_{B/A}$ regioisomers of *N*-butylPP, *N*-pentylPP, *N*-hexylPP, and *N*-isobutylPP were found to be less potent as ferrochelatase inhibitors than the N_A plus N_B regioisomers of *N*-ethylPP (Fig.

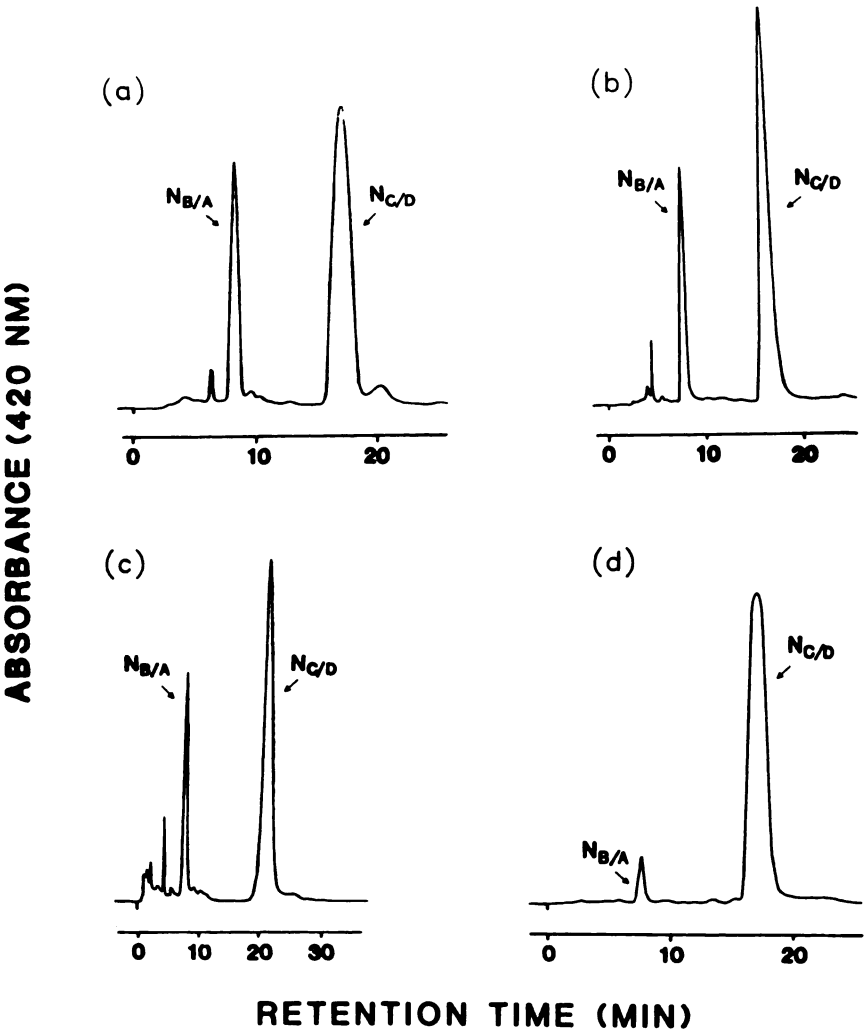

Fig. 5. High performance liquid chromatography analysis of the *N*-alkylporphyrin dimethyl esters isolated from the livers of (a) 4-butyl-DDC-, (b) 4-pentyl-DDC-, (c) 4-hexyl-DDC-, and (d) 4-isobutyl-DDC-treated rats. A Partisil-10-PAC column (4.6 × 250 mm), eluted with hexane/tetrahydrofuran/methanol, 48.5:48.5:3.0 (v/v/v), and a variable wavelength detector, set at 420 nm, were used for the analysis.

TABLE 2

Percentage of $N_{B/A}$ regioisomers in the *N*-alkylPP isolated from phenobarbital-pretreated rats after the administration of DDC analogues

DDC Analogues	$N_{A/B}$ Regioisomers in *N*-AlkylPP
	%
4-Butyl	25.9 ± 5.3
4-Pentyl	19.2 ± 4.2
4-Hexyl	22.8 ± 2.9
4-Isobutyl	3.8 ± 2.2

6, a, b, c, and d). Therefore the decrease in ferrochelatase-lowering activity as the length and bulk of the 4-alkyl substituent of DDC analogues increases may be explained by 1) the formation of a smaller proportion of the active N_A and/or N_B regioisomers and more of the much less active N_C and/or N_D regioisomers and 2) decreased potency, as ferrochelatase inhibitors, of $N_{B/A}$ regioisomers of *N*-butylPP, *N*-pentylPP, *N*-hexylPP, and *N*-isobutylPP relative to the $N_{B/A}$ regioisomers of *N*-methylPP and *N*-ethylPP.

The final question that we would like to address is the following: why does the ratio of N_A and/or N_B regioisomers in the regioisomer mixture of an *N*-alkylPP decrease as the length and bulk of the 4-alkyl substituent of a DDC analogue increases? The following studies may throw some light on this problem. De Matteis *et al.* (19) showed that the regioisomer composition of *N*-ethylPP depended on the hepatic cytochrome P-450 isozyme(s) that predominated when 4-ethyl-DDC was administered to rats. Thus, after pretreatment of rats with phenobarbital the N_A regioisomer of *N*-ethylPP predominated (63% of the total regioisomers) whereas after β-naphthoflavone pretreatment the N_D regioisomer predominated (51% of the total regioisomers). De Matteis *et al.* (19) suggested that the apoprotein of a cytochrome P-450 isozyme may specifically direct the alkyl group ejected from the 4-position of the dihydropyridine onto one of the four pyrrole nitrogens. In our studies the proportion of $N_{B/A}$ regioisomers decreases drastically with an increase in the size and bulk of the 4-alkyl substituent of DDC. This suggests that DDC analogues with bulky 4-alkyl substituents may have different isozyme specificity than does 4-ethyl-DDC; these isozymes may direct alkylation primarily to the C and D ring nitrogens of the heme moiety of cytochrome P-450. An alternative interpretation of our data is the following: The same cytochrome P-450 isozyme or isozymes may be involved in interaction with the dihydropyridines despite differences in the size of the 4-alkyl group. The size of the 4-alkyl group would influence the orientation of binding of the dihydropyridine at the active site and thus account for differences in the regioisomers formed.

To resolve these questions it will be necessary to purify the

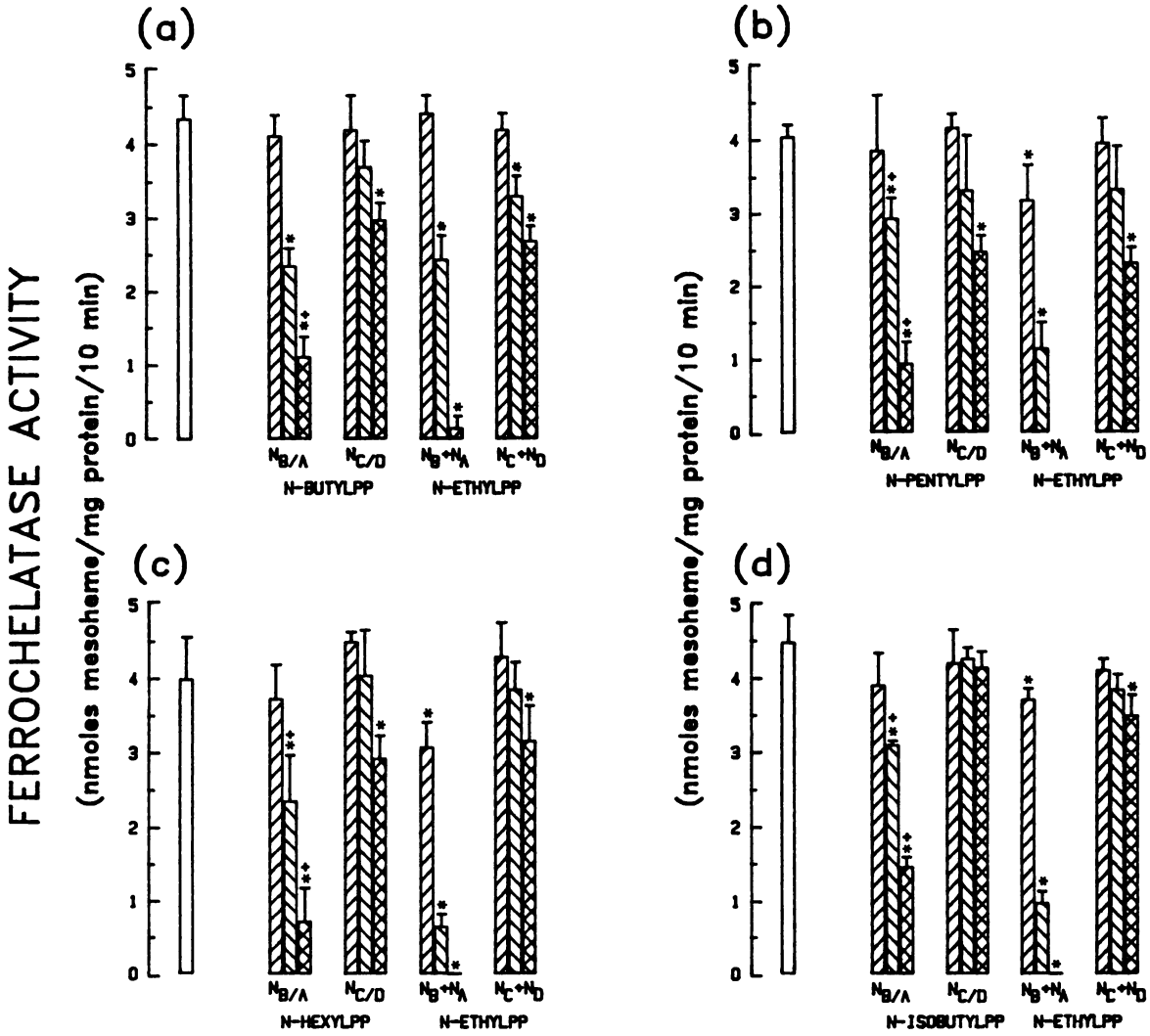

Fig. 6. Ferrochelatase activity of chick embryo liver cell culture after incubation with regioisomers of (a) *N*-butylPP, (b) *N*-pentylPP, (c) *N*-hexylPP, and (d) *N*-isobutylPP ▨, 3.4 pmol/ml; ▧, 34 pmol/ml; ⊠, 340 pmol/ml; □, control. Each *bar* represents the mean of four determinations (± standard deviation); results were confirmed by two additional experiments. *Significantly different from control as determined by a one-way analysis of variance ($p \leq 0.05$) and a Newman-Keuls test ($p \leq 0.05$). †Significantly different from the same dose and regioisomers of synthetic *N*-ethylPP.

major isozymes of cytochrome P-450. After reconstituting active cytochrome P-450 monooxygenase preparations with the purified isozymes, the nature of the *N*-alkyl regioisomers formed after mechanism-based inactivation by DDC analogues must be determined. Studies of this type should be helpful in elucidating the characteristics of the active site of cytochrome P-450 isozymes.

References

1. Tephly, T. R., A. H. Gibbs, and F. De Matteis. Studies on the mechanism of experimental porphyria produced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine: role of a porphyrin-like inhibitor of protohaem ferrolyase. *Biochem. J.* **180:**241–244 (1979).
2. De Matteis, F., A. H. Gibbs, and T. R. Tephly. Inhibition of protohaem ferrolyase in experimental porphyria: isolation and partial characterization of a modified porphyrin inhibitor. *Biochem. J.* **188:**145–152 (1980).
3. Ortiz de Montellano, P. R., H. S. Beilan, and K. L. Kunze. *N*-Methylprotoporphyrin IX: chemical synthesis and identification as the green pigment produced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine treatment. *Proc. Natl. Acad. Sci. U.S.A.* **78:**1490–1494 (1981).
4. Ortiz de Montellano P. R., H. S. Beilan, and K. L. Kunze. *N*-Alkylprotoporphyrin IX formation in 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated rats: transfer of the alkyl group from the substrate to the porphyrin. *J. Biol. Chem.* **256:**6708–6713 (1981).
5. Ortiz de Montellano, P. R., K. L. Kunze, S. P. C. Cole, and G. S. Marks. Inhibition of hepatic ferrochelatase by the four isomers of *N*-methylprotoporphyrin IX. *Biochem. Biophys. Res. Commun.* **97:**1436–1442 (1980).
6. McCluskey, S. A., E. P. Sutherland, W. J. Racz, and G. S. Marks. Isolation of an *N*-alkylprotoporphyrin IX from chick embryo livers following the administration of 3,5-diethoxycarbonyl-1,4-dihyro-4-ethyl-2,6-dimethylpyridine. *Can. J. Physiol. Pharmacol.* **65:**1500–1502 (1987).
7. Ortiz de Montellano, P. R., K. L. Kunze, S. P. C. Cole, and G. S. Marks. Differential inhibition of hepatic ferrochelatase by the isomers of *N*-ethylprotoporphyrin IX. *Biochem. Biophys, Res. Commun.* **103:**581–586 (1981).
8. Marks, G. S., D. T. Allen, C. T. Johnston, E. P. Sutherland, K. Nakatsu, and R. A. Whitney. Suicidal destruction of cytochrome P-450 and reduction of ferrochelatase activity by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine and its analogues in chick embryo liver cells. *Mol. Pharmacol.* **27:**459–465 (1985).
9. De Matteis, F., A. H. Gibbs, and C. Harvey. Studies on the inhibition of ferrochelatase by *N*-alkylated dicarboxylic porphyrins: steric factors involved and evidence that the inhibition is reversible. *Biochem. J.* **226:**537–544 (1985).
10. McCluskey, S. A., G. S. Marks, E. P. Sutherland, N. Jacobsen, and P. R. Ortiz de Montellano. Ferrochelatase-inhibitory activity and *N*-alkylprotoporphyrin formation with analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) containing extended 4-alkyl groups: implications for the active site of ferrochelatase. *Mol. Pharmacol.* **30:**352–357 (1986).
11. Augusto, O., H. S. Beilan, and P.R. Ortiz de Montellano. The catalytic mechanism of cytochrome P-450: spin-trapping evidence for one electron substrate oxidation. *J. Biol. Chem.* **257:**11288–11295 (1982).
12. Cole, S. P. C., R. A. Whitney, and G. S. Marks. Ferrochelatase-inhibitory and porphyrin-inducing properties of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-

trimethylpyridine and its analogues in chick embryo liver cells. *Mol Pharmacol.* **20**:395–403 (1981).
13. De Matteis, F., A. H. Gibbs, P. B. Farmer, and J. H. Lamb. Liver production of *N*-alkylated porphyrins caused in mice by treatment with substituted dihydropyridines: evidence that the alkyl group on the pyrrole nitrogen atom originates from the drug. *FEBS Lett.* **129**:328–331 (1981).
14. Ortiz de Montellano, P. R., G. S. Yost, B. A. Mico, S. E. Dinizo, M. A. Correia, and H. Kumbara. Destruction of cytochrome P-450 by 2-isopropyl-4-pentenamide and methyl 2-isopropyl-4-pentenoate: mass spectrometric characterization of the prosthetic heme adducts and nonparticipation of epoxide metabolites. *Arch. Biochem. Biophys.* **197**:524–533 (1979).
15. Morgan, R. O., P. W. F. Fischer, J. K. Stephens, and G. S. Marks. Thyroid hormone enhancement of drug-induced porphyrin biosynthesis in chick embryo liver cells maintained in serum-free Waymouth medium. *Biochem. Pharmacol.* **25**:2609–2612 (1976).
16. Cole, S. P. C., E. J. Vavasour, and G. S. Marks. Drug-induced porphyrin biosynthesis. XIX. Potentiation of the porphyrin-inducing effects of SKF 525-A in the chick embryo liver by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, an inhibitor of ferrochelatase. *Biochem. Pharmacol.* **28**:3533–3538 (1979).
17. Falk, J. E. *Porphyrins and Metalloporphyrins.* Elsevier, Amsterdam, 236 (1964).
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
19. De Matteis, F., A. H. Gibbs, and C. Hollands. *N*-Alkylation of the haem moiety of cytochrome P-450 caused by substituted dihydropyridines: preferential attack of different pyrrole nitrogen atoms after induction of various cytochrome P-450 isoenzymes. *Biochem. J.* **211**:455–461 (1983).
20. Marks, G. S., J. Powles, M. Lyon, S. McCluskey, E. Sutherland, and D. Zelt. Patterns of porphyrin accumulation in response to xenobiotics: parallels between results in chick embryos and rodents. *Ann. N.Y. Acad. Sci.* **514**:113–127 (1987).

Send reprint requests to: Dr. Gerald S. Marks, Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario K7L 3N6, Canada.