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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

S. A. McCLUSKEY, G. S. MARKS, E. P. SUTHERLAND, N. JACOBSEN, and P. R. ORTIZ DE MONTELLANO

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

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SUMMARY

The ferrochelatase-inhibitory activity, porphyrin-inducing activity, and cytochrome P-450- and heme-destructive effects of a variety of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) were studied in chick embryo liver cells. The ferrochelatase-inhibitory activity of the 4-butyl, 4-pentyl, 4-hexyl, and 4-cyclopropylmethyl analogues of DDC was considered to be due to the formation of the corresponding *N*-alkylporphyrins. These *N*-alkylporphyrins were isolated from the livers of phenobarbital-pretreated rats following administration of the corresponding DDC analogues. The 4-isobutyl analogue did not have ferrochelatase-inhibitory activity despite its ability to cause formation of an *N*-isobutylporphyrin in rat liver. The 4-chloromethyl analogue of DDC inhibited ferrochelatase activity. The inability to isolate an *N*-alkylporphyrin from rat liver with this analogue may be due to its lability. The porphyrin-inducing activity of these analogues depended on their ferrochelatase-inhibitory potency and lipophilicity. The DDC analogues caused cytochrome P-450 and heme destruction. The relative ferrochelatase-inhibitory activity of the DDC analogues has implications for a postulated model of the binding of porphyrins in the ferrochelatase active site.

The dihydropyridine, DDC (I, Table 1), which has porphyrinogenic activity in a variety of animal species (1), has served as a valuable tool in the study of the control of heme biosynthesis. DDC, when administered to rodents, causes the accumulation in the liver of *N*-MePP (2–4), a potent inhibitor of ferrochelatase (EC 4.99.1.1) activity (2, 3, 5). The source of the *N*-methyl substituent of *N*-MePP is the 4-methyl group of DDC (6, 7). DDC and several 4-alkyl analogues of DDC serve as suicide substrates for cytochrome P-450 (8). According to current ideas a radical cation is produced by the one-electron oxidation of the nitrogen atom of DDC by cytochrome P-450, and this is followed by ejection of the 4-alkyl radical which alkylates the heme prosthetic group. The source of the protoporphyrin IX moiety of *N*-MePP is therefore inferred to be the prosthetic heme of cytochrome P-450 (8).

The four regioisomers of *N*-MePP (Fig. 1a) are equally potent as inhibitors of ferrochelatase from rat liver and from cultured chick embryo liver cells (5). Since the regioisomers (Fig. 1a) differ in the location of the methyl group on the porphyrin ring and in the region of the molecule which is tilted out of the porphyrin plane by the *N*-alkyl group (9, 10), a relatively nondiscriminating protoporphyrin-binding site was suggested for ferrochelatase. This conclusion was modified by a study of the regioisomers of *N*-ethylprotoporphyrin IX. The two regioisomers of *N*-ethylprotoporphyrin IX (Fig. 1b) with *N*-ethyl groups on rings A and B inhibited ferrochelatase as effectively (11) as the four regioisomers of *N*-MePP (Fig. 1a), whereas the regioisomers of *N*-ethylprotoporphyrin IX with *N*-ethyl groups on rings C and D were 30–100 times less potent. The steric constraints on the *N*-alkyl group are therefore more marked when the group is located on the propionic acid-substituted pyrrole rings than on the vinyl-substituted rings. Since the protoporphyrin-binding site on ferrochelatase 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 groups on rings A and B may be compatible with inhibitory activity. This idea is supported by the finding that 4-propyl DDC (II, Table 1) has ferrochelatase-inhibitory activity when tested in chick embryo liver cell culture (12, 13). The first objective of this study was to determine how large a group was

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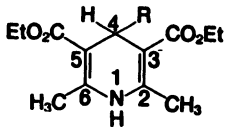
ABBREVIATIONS: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; *N*-MePP, *N*-methylprotoporphyrin IX; EDTA, ethylenediaminetetraacetate; ALAS, δ-aminolevulinic acid synthetase.

TABLE 1
Structure of the dihydropyridine analogues of DDC



Analogue	R	Analogue	R
I	CH_3	V	C_6H_{13}
II	C_3H_7	VI	$—CH_2CH(CH_3)_2$
III	C_4H_9	VII	$—CH_2Cl$
IV	C_5H_{11}	VIII	$—CH_2—◁$

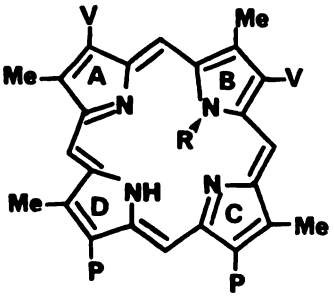


Fig. 1. (a) *R* = methyl, one of the four possible regioisomers of *N*-MePP. The other isomers have an *N*-methyl group on pyrrole rings, A, C or D. (b) *R* = ethyl, one of the four possible regiosiomers of *N*-ethylprotoporphyrin IX. The other isomers have an *N*-ethyl group on pyrrole rings A, C, or D. *V*, vinyl; *P*, propionic acid; *Me*, methyl.

compatible with inhibitory activity. For this reason the 4-butyl (**III**), 4-pentyl (**IV**), 4-hexyl (**V**), 4-isobutyl (**VI**) and 4-cyclopropylmethyl (**VIII**) analogues of DDC (Table 1) were synthesized and tested for ferrochelatase-inhibitory activity, porphyrin-inducing activity, and suicidal destruction of cytochrome P-450 in chick embryo liver cells.

The second objective of our study was to determine in chick embryo liver cells the effects on ferrochelatase activity, porphyrin-inducing activity, and cytochrome P-450 of a DDC analogue containing a 4-alkyl group with a leaving group, *viz.*, 4-chloromethyl DDC (**VII**, Table 1). The transfer of the 4-chloromethyl group to a pyrrole nitrogen of the heme in cytochrome P-450 might be expected to lead to a second alkylation either by direct displacement or by elimination-addition. Bridged porphyrins of this type have been prepared for octaethylporphyrin or diiodomethane (14).

The third objective was to attempt to isolate, purify, and identify *N*-alkylporphyrins after administration of the dihydropyridines **III**, **IV**, **V**, **VII**, and **VIII** (Table 1) to phenobarbital-pretreated rats. For this part of the study rats rather than chick embryos were used in order to obtain sufficient liver for isolation, purification, and identification of the *N*-alkylporphyrins.

Methods

Source of compounds. DDC (**I**) and 4-isobutyl DDC (**VI**) were synthesized as described previously (8, 12).

4-Chloromethyl DDC (VII). 4-Chloromethyl DDC was synthesized by the procedure of Brignell *et al.* (15) from ethyl α-aminocrotonate (77.4 mmol), 1,2-dichloroethyl ethyl ether (81.8 mmol), and 4.7% (w/w) aqueous ammonia (60 ml). Crystallization of the product from ethanol/water afforded the product as fine yellow crystals, m.p. 133–134°, λ_{max} 350 and 234 (ε 8,277 and 20,397). Brignell *et al.* (15) reported m.p. 134–136°, λ_{max} 349 and 231 (ε 7,650 and 19,400); 240 MHz ^{1}H-NMR ($CDCl_3$): 1.30 (t, 6H, J = 7.1 Hz, $—CH_2CH_3$), 2.33 (s, 6H, $—CH_3$), 3.55 (d, 2H, J = 4.2 Hz, $—CH_2Cl$), 4.18 and 4.22 (AB of ABX_3, 4H, J_{AB} = 10.8, $J_{AX} = J_{BX}$ = 7.1 Hz, $—OCH_2—$), 4.32 (br.t, 1H, J = 4.0 Hz, $CH_2Cl—CH—$), 5.69 ppm (br.s, 1H, NH); mass spectrum *m/z* 299 ($M - H_2$, 0.1%), 265 ($M - HCl$, 18%), 252 (100%), 224, 220, 196, 192, 77.

4-Cyclopropylmethyl DDC (VIII). 2-Cyclopropylacetaldehyde (16) was generated from its diethylacetal (4.39 mmol) by treatment with 0.5 M HCl (14 ml) for 10 min at room temperature. After neutralization with saturated sodium bicarbonate (5.3 ml), ethyl acetoacetate (9.57 mmol), pulverized ammonium carbonate (21.9 mmol), and water (1.5 ml) were added. The flask was purged with nitrogen, capped, and stirred in the dark at 21° for 7 days. The yellow precipitate was washed with water and recrystallized twice from ethanol/water, yielding white needles, m.p. 113.5–114.5°; λ_{max} 352 and 238 (ε 7,519 and 15,875); 240 MHz ^{1}H-NMR ($CDCl_3$): −0.08 and 0.30 (AA^1MM^1 of AA^1MM^1X, 4H, $J_{AX} = J_{A^1X}$ = 4.8 Hz, $J_{MX} = J_{M^1X}$ = 8.0 Hz, cyclopropyl $—CH_2—$), 0.60 (m, 1H, cyclopropyl CH), 1.21 (br.t., 2H, J = 6.4 Hz, $—CH-CH_2—$), 1.28 (t, 6H, J = 7.1 Hz, OCH_2CH_3), 2.27 (s, 6H, $—CH_3$), 4.02 (t, 1H, J = 6.0 Hz, ring CH), 4.14 and 4.18 (AB of ABX_3, 4H, J_{AB} = 10.8 Hz, $J_{AX} = J_{A^1X}$ = 7.1 Hz, $OCH_2—$), 5.64 ppm (br.s, 1H, NH); mass spectrum *m/z* 307 (M^+, 0.2%), 306 (0.8%), 305 (3%) 252, 251, 206 (100%), 205, 178, 150, 106.

4-Butyl DDC (III), 4-pentyl DDC (IV), and 4-hexyl DDC (V). These dihydropyridines were synthesized by the procedure of Loev and Snader (17). A solution of ethyl acetoacetate (0.20 mol), concentrated NH_4OH (0.1 mol), and the appropriate aldehyde (0.10 mol) in 60 ml of ethanol was refluxed for 3 hr. Upon cooling, the solution was added to water at 0° (160 ml). In the case of the 4-butyl and 4-pentyl analogues of DDC, an oil separated, which solidified and was separated by filtration. The crude material was purified by successive crystallizations from ethanol/water and hexane.

In the case of the 4-hexyl analogue of DDC, an oil separated which would not solidify. The oil was therefore dissolved in ether, and the ether layer separated from the aqueous layer and was washed successively with 1 N hydrochloric acid and water. The ether solution was dried ($MgSO_4$) and evaporated to dryness, and the residue was crystallized twice from hexane. The elemental analyses of the dihydropyridines were in accordance with their structures. The dihydropyridines had the following properties.

4-Hexyl DDC. m.p. 55.5–56.5° [Jaeckle (18) reported m.p. 54°]; UV (ethanol), λ_{max} 352 and 233 (ε 7,386 and 16,285); 80 MHz ^{1}H-NMR ($CDCl_3$): 5.45δ (NH, 1H, broad singlet), 4.18δ (OCH_2, 4H, quartet, J = 8.0 Hz), 2.28δ (2,6-CH_3, s); mass spectrum *m/z* 337 (M^+, 0.03%), 308, 306, 292, 252 (M−hexyl, base, 100%), 224, 206, 196, 179, 150, 134, 106, 77.

4-Pentyl DDC. m.p. 67.5–70.0°; UV (ethanol) λ_{max} 349 and 233 (ε 7,386 and 16,582); 80 MHz ^{1}H-NMR ($CDCl_3$): 5.48δ (NH, 1H, broad s), 4.18δ (OCH_2, 4H, q, J = 7.32 Hz, 2.28δ (2,6-CH_3, 6H, s); mass spectra *m/z* 323 (M^+, 0.03%) 278, 252 (M−pentyl, base, 100%), 224, 206, 196, 179, 150, 134, 106, 77.

4-Butyl DDC. m.p. 96–97.5°; UV (ethanol), λ_{max} 354 and 233 (ε 7,320 and 17,005); 80 MHz ^{1}H-NMR ($CDCl_3$): 5.47δ (NH, 1H, broad s), 4.19δ (OCH_2, 4H q, J = 7.33 Hz), 2.28δ (2,6-CH_3, 6H, s); mass spectra *m/z* 309 (M^+, 0.06%), 264, 252 (M−butyl, base, 100%), 224, 206, 196, 179, 150, 134, 106, 77.

Porphyrins and porphyrin methyl esters were purchased from Porphyrin Products (Logan, UT).

Determination of ferrochelatase activity. The details of the cell culture technique have been described previously (13, 19). After an initial incubation period of 24 hr, the medium was discarded and replaced with fresh medium. After a further 24-hr incubation period, a DDC analogue (Table 1), dissolved in 95% ethanol, was added to the dishes (maximum total volume added, 20 μl). Ferrochelatase activity was assayed 6 hr after addition of a DDC analogue as described

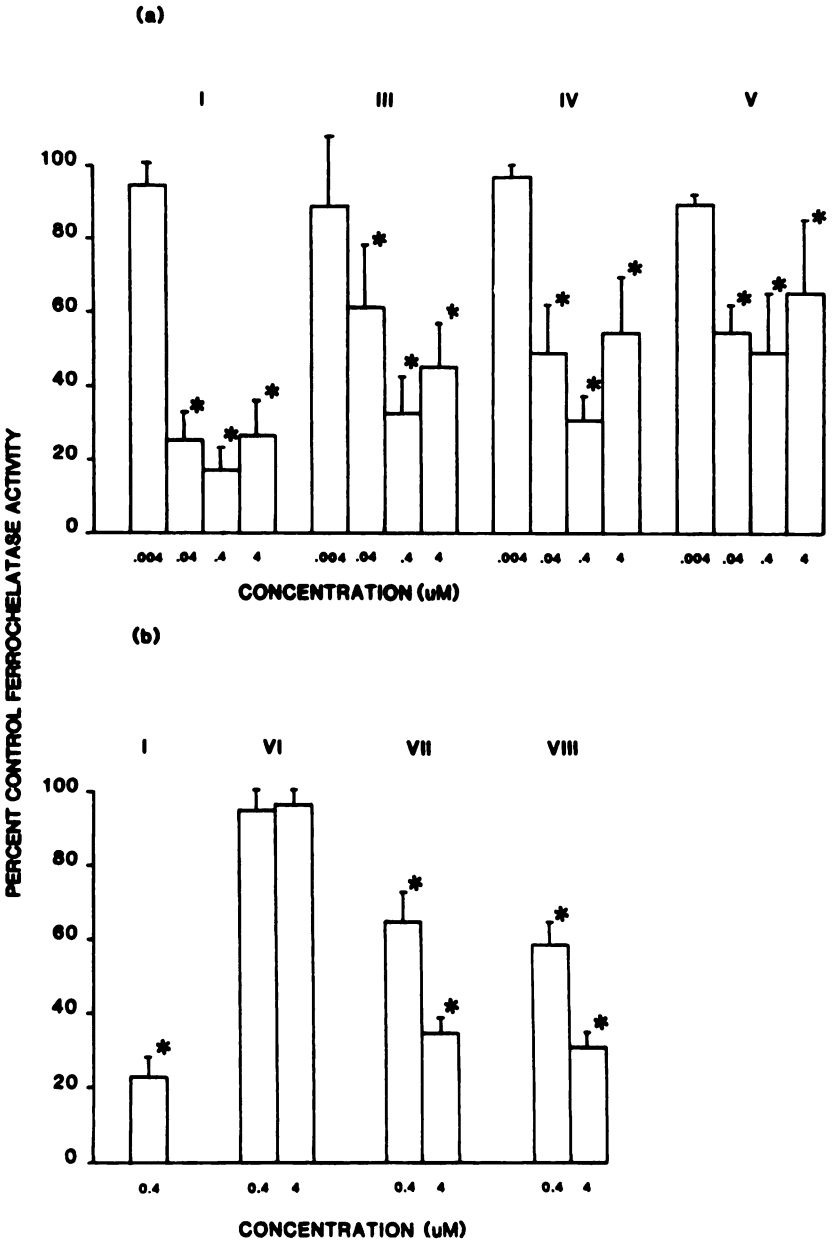


Fig. 2. Percentage of control ferrochelatase activity 6 hr after the addition of DDC and analogues to cell culture: a, I, DDC; III, 4-butyl DDC; IV, 4-pentyl DDC; V, 4-hexyl DDC. b, I, DDC; VI, 4-isobutyl DDC; VII, 4-chloromethyl DDC; VIII, 4-cyclopropylmethyl DDC. Each *bar* represents the mean of 9–12 determinations ± standard deviation in four experiments. *, significantly different from control, $p \leq 0.05$, Newman-Keuls test. Control ferrochelatase activity, 3.5 ± 0.9 nmol of mesoheme/10 min/mg of protein.

TABLE 2

Electronic absorption spectra of the zinc complexes and zinc-free neutral *N*-alkyl porphyrin dimethyl esters isolated from the livers of phenobarbital-pretreated rats following administration of DDC analogues

DDC analogues	Absorption maxima								
	Zinc complex				Neutral absorption spectrum				
	Soret	β	α	α′	Soret	IV	III	II	I
	nm								
4-Butyl (III)	432	546	590	634	418	514	546	592	
4-Pentyl (IV)	432	544	590	632	414	512	544	592	656
4-Hexyl (V)	432	546	592	634	416	512	546	594	652
4-Cyclopropylmethyl (VIII)	430	546	594	632					

previously (12, 13, 20) by a modification of the pyridine hemochromogen method (21) using mesoporphyrin and ferrous iron as substrates.

Determination of total porphyrins. For the determination of total porphyrins, chick embryo liver cells were maintained in 6-cm diameter dishes containing 5 ml of medium. Drugs were dissolved in 95% ethanol for addition to the dishes (maximal total volume 15 μl). Total porphyrins were measured 24 hr after addition of the drugs (22).

***In vitro* cytochrome P-450 and heme destruction.** The method used (13) was adapted from that used by Omura and Sato (23) for the preparation of rat liver microsomes. The livers of 18-day-old chick embryos were used for the preparation of a microsomal suspension in 0.1 M potassium phosphate buffer, pH 7.4, containing 1.5 mM EDTA. The suspension contained 2 mg of microsomal protein/ml. A DDC analogue (500 μg) dissolved in ethanol (20 μl) was added to an Erlen-

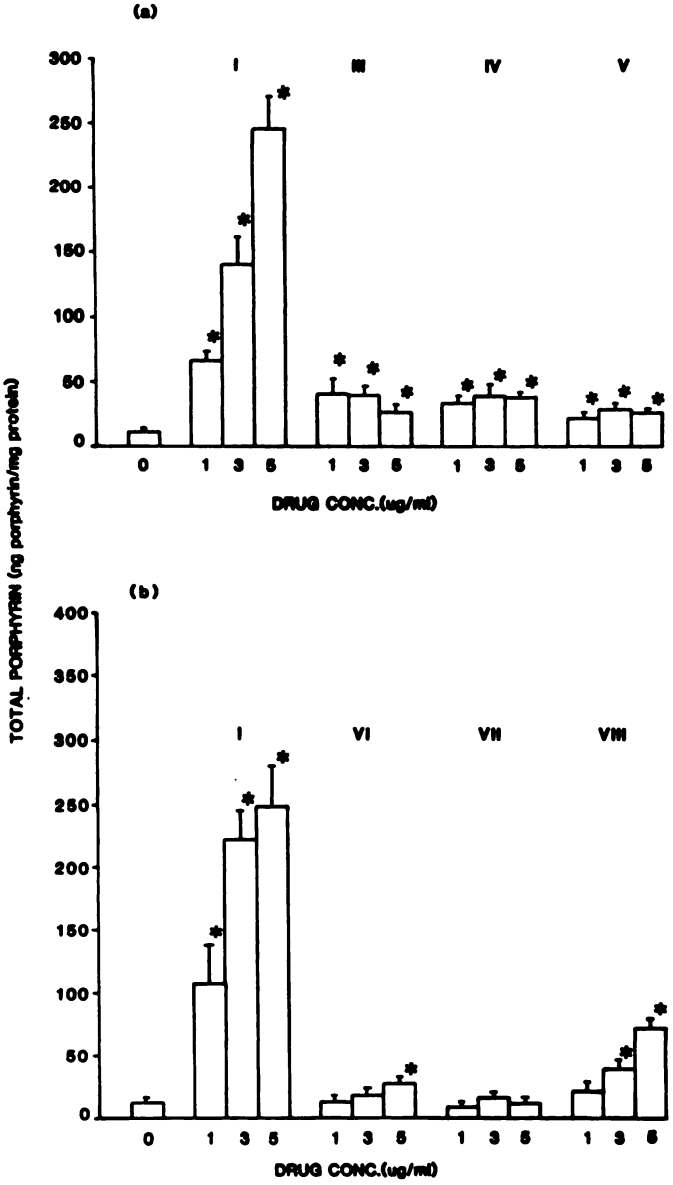


Fig. 3. Porphyrin accumulation 24 hr after the addition of DDC analogues to cell culture: a, I, DDC; III, 4-butyl DDC; IV, 4-pentyl DDC; V, 4-hexyl DDC. b, I, DDC; VI, 4-isobutyl DDC; VII, 4-chloromethyl DDC; VIII, 4-chloropropylmethyl DDC. Each *bar* represents the mean of five determinations ± standard deviation in one experiment; the results were confirmed in two additional experiments. *, significantly different from control, $p \leq 0.05$, Newman-Keuls test.

meyer flask (25 ml) containing microsomal suspension (2 ml) and a 2 mM solution of NADPH in 0.1 M potassium phosphate buffer, pH 7.4, containing EDTA (1.5 mM) (2 ml). After incubating the mixture at 37° for 30 min in a shaking water bath, the reaction was stopped by cooling the flasks on ice. After removing an aliquot (1.5 ml) for subsequent heme determination, a few mg of sodium dithionite were added to the remaining 2.5 ml of the reaction mixture. The reaction mixture was then distributed between the reference and sample cuvettes of a Unicam SP8-100 spectrophotometer, and the quantity of cytochrome P-450 was measured in the usual manner. The following controls were run: (a) omission of DDC analogue, (b) omission of NADPH, and (c) omission of DDC analogue and NADPH.

To the 1.5-ml aliquot of reaction mixture set aside for heme determination, pyridine (0.75 ml) and 0.25 N NaOH (1.5 ml) were added and the mixture was immediately distributed between sample and reference cuvettes. A few mg of sodium dithionite were added to the sample cuvette and 17 μl of 3 mM potassium ferricyanide were added to the reference cuvette. The quantity of heme was calculated using the difference between the absorbance of the peak at 557 nm and the absorbance at 575 nm; an extinction coefficient of 32.4 cm^{-1} mM^{-1} was used (23).

Protein determination. Protein was assayed by the method of Lowry *et al.* (24).

Statistical analysis. Student's *t* test was used to determine whether two means were significantly different from each other ($p \leq 0.05$). This test was employed for the cytochrome P-450 and heme studies (see Table 3). When it is necessary to compare more than two means (e.g., in a study of the effects of several doses of a DDC analogue on ferrochelatase activity), one-way analysis of variance ($p < 0.05$) was employed. If a significant *F* ratio at the 0.05 level was obtained, a Newman-Keuls test was used to determine which means were significantly different from each other ($p \leq 0.05$).

Isolation and purification of *N*-alkylporphyrins (7). Sprague-Dawley male rats weighing approximately 300 g received aqueous sodium phenobarbital (80 mg/kg daily) intraperitoneally for 4 days prior to injection by the same route of a DDC analogue (400 mg/kg) in dimethyl sulfoxide (100 mg/ml). The rats were decapitated 4 hr after injection of a DDC analogue and their livers were perfused *in situ* with 1.15% potassium chloride. The livers were then removed and rinsed several times with ice-cold 1.15% potassium chloride. After draining, the livers were homogenized in a Waring blender with ice-cold 5% (v/v) H_2SO_4/methanol (100 ml/liver), and the mixture was stored in the dark at 4° for 17–20 hr. The mixture was filtered, diluted with an equal volume of water, and extracted twice with methylene chloride. The methylene chloride solution was washed three times with water, dried (sodium sulfate), and evaporated to approximately 20 ml. When four rats were used, a solution of zinc acetate (22 μmols) dissolved in methanol (1.5 ml) was added, and the mixture was evaporated to dryness. Correspondingly larger amounts of zinc acetate were used when larger numbers of rats were used. The residue was dissolved in acetone (approximately 1 ml) and purified by thin layer chromatography on Analtech (2000 μm) silica gel G plates developed with methylene chloride/acetone, 4:1 (v/v). The red fluorescing band (R_f 0.67–0.97) was extracted with acetone and was rechromatographed on a 500-μm Analtech silica gel G plate using chloroform/acetone, 10:1 (v/v) as developing solvent. A green band which fluoresced red under long wavelength light (R_f 0.46–0.52) was eluted with acetone and its spectral characteristics were determined. Recovery experiments were performed by adding 25–125 μg of *N*-MePP to rat livers. The recovery was found to be 25.3 ± 6.5%. The Zn *N*-alkylporphyrin dimethyl ester was purified by high pressure liquid chromatography on a Partisil-10 PAC column (4.6 × 250 mm). A 20-min linear gradient (0–100%) of methanol into hexane/tetrahydrofuran, 1:1 (v/v), was used for elution. After recording the electronic absorption spectrum in acetone, the zinc cation was removed by treatment with 5% H_2SO_4 in methanol as previously described (25) and the electronic absorption spectrum was recorded in acetone. In addition, the field desorption mass spectra of the *N*-pentyl and *N*-hexyl protoporphyrin IX dimethyl esters were run by the Mass Spectrometry Resource, School of Pharmacy, University of California.

Results and Discussion

The ferrochelatase-inhibitory activities of DDC analogues (Table 1) were compared at a series of concentrations in the chick embryo liver cell culture medium (Fig. 2). The 4-butyl (III), 4-pentyl (IV), and 4-hexyl (V) analogues had ferrochelatase-inhibitory activities which were significantly different from that of DDC at a concentration of 0.04 μM (Fig. 2a). The ferrochelatase-lowering potency of the highest concentrations (4.0 μM) of the 4-alkyl analogues of DDC (Fig. 2a) tends to be lower than the 0.4 μM dose, although this is not statistically significant. Cole *et al.* (12) noted a similar effect with DDC analogues. A possible explanation for these results is that high doses of DDC and analogues result in levels of ALAS induction sufficient to elevate protoporphyrin IX to levels that compete

TABLE 3

In vitro levels of chick embryo hepatic microsomal cytochrome P-450 and heme following incubation with DDC analogues

Compound	Compound no.	Dose	Cytochrome P-450[a]	Heme[a]	Loss of cytochrome P-450	Loss of heme
		μg/ml	nmol/mg protein	nmol/mg protein	nmol/mg protein	nmol/mg protein
4-Butyl	III	0	0.278 ± 0.014	0.640 ± 0.049		
		125	0.148 ± 0.023[b]	0.473 ± 0.036[b]	0.130	0.167
4-Pentyl	IV	0	0.278 ± 0.024	0.640 ± 0.049		
		125	0.169 ± 0.009[b]	0.497 ± 0.016[b]	0.109	0.143
4-Hexyl	V	0	0.278 ± 0.014	0.640 ± 0.049		
		125	0.163 ± 0.005[b]	0.505 ± 0.018[b]	0.115	0.135
4-Isobutyl	VI	0	0.278 ± 0.024	0.640 ± 0.049		
		125	0.181 ± 0.011[b]	0.540 ± 0.016[b]	0.097	0.100
4-Chloromethyl	VII	0	0.294 ± 0.004	0.687 ± 0.039		
		125	0.181 ± 0.019[b]	0.578 ± 0.007[b]	0.113	0.109
4-Cyclopropylmethyl	VIII	0	0.294 ± 0.004	0.687 ± 0.039		
		125	0.187 ± 0.008[b]	0.562 ± 0.024[b]	0.107	0.125

[a] Values given are means (± standard deviation) for four determinations.
[b] Significantly different from control, $p \leq 0.05$, Student's t test.

effectively with the *N*-alkylprotoporphyrins for the active site of ferrochelatase. The 4-cyclopropylmethyl (**VIII**) and the 4-chloromethyl (**VII**) analogues were shown to have ferrochelatase-inhibitory activity but were less potent than DDC (Fig. 2). The 4-isobutyl analogue (**VI**) was shown to be inactive. The ferrochelatase-inhibitory activity of the 4-butyl (**III**), 4-pentyl (**IV**), 4-hexyl (**V**), and 4-cyclopropylmethyl (**VIII**) analogues is considered to be due to the formation of the corresponding *N*-alkylporphyrins. These *N*-alkylporphyrins were isolated from the livers of phenobarbital-pretreated rats following administration of the corresponding DDC analogues. The *N*-alkylporphyrins were identified by the electronic absorption spectra of their zinc complexes and zinc-free neutral dimethyl esters (Table 2) since these spectra were closely similar to those previously reported for the corresponding porphyrins obtained with DDC and the 4-ethyl analogue of DDC (4, 7). The field desorption mass spectra of the porphyrins obtained with 4-pentyl (**IV**) and the 4-hexyl (**V**) analogues provides further evidence for their structure. The monoprotonated molecular ions of the porphyrins (m/z 661 and 674, respectively) are those expected for structures constructed from the dimethyl ester of protoporphyrin IX and the pentyl and hexyl moieties.

In view of the ferrochelatase-inhibitory activity of the 4-chloromethyl analogue (**VII**) (Fig. 2), it was surprising that an *N*-alkylporphyrin could not be isolated from phenobarbital-pretreated rat liver. It is possible that the *N*-alkylporphyrin formed from this analogue, which is expected to be more labile than *N*-alkylporphyrins formed from analogues, **III, IV, V,** and **VIII,** did not survive the treatment of liver with 5% H_2SO_4/methanol. The inability of the 4-isobutyl analogue (**VI**) to reduce ferrochelatase activity (Fig. 2) is interesting in view of the fact that this analogue has been shown (8) to cause the formation of an *N*-alkylprotoporphyrin in phenobarbital-pretreated rat liver, tentatively identified by electronic absorption spectra and field desorption mass spectrum as *N*-isobutylprotoporphyrin IX dimethyl ester.

DDC, 4-butyl (**III**), 4-pentyl (**IV**), 4-hexyl (**V**), and 4-isobutyl (**VI**) analogues were tested for ALAS-inducing activity 6 hr after their addition to chick embryo liver cells (26). ALAS activity was found to be 5–10 times above control (results not shown). DDC and related analogues owe their porphyrinogenic action to a combination of two effects: (a) ferrochelatase inhibition via *N*-alkylprotoporphyrin IX formation and (b) a second mechanism, whereby an elevation of ALAS (EC 2.3.1.37) is induced, independent of inhibition of heme biosynthesis or stimulation of heme degradation. Two ways whereby this might occur are: 1) a direct action on the nucleus to increase the amount of an mRNA for ALA-S, and 2) increased synthesis of the mRNA for apo-cytochrome P-450, leading to increased synthesis of apo-cytochrome P-450 with increased utilization of heme from the "regulatory heme pool" for the synthesis of cytochrome P-450 (27, 28). It has been pointed out that all drugs that induce ALAS by this second mechanism are lipophilic (28). The 4-isobutyl analogue (**VI**) has greater lipophilicity than does DDC (29) but, unlike DDC, does not possess ferrochelatase-inhibitory activity. The fact that it is less potent as a porphyrin-inducing drug than DDC (Fig. 3) may be attributed to its lack of ferrochelatase-inhibitory activity. The lower porphyrinogenic potency of the lipophilic 4-butyl (**III**), 4-pentyl (**IV**), 4-hexyl (**V**), 4-chloromethyl (**VII**), and 4-cyclopropylmethyl (**VIII**) analogues than of DDC may be attributed to their lower potency as ferrochelatase-inhibitory agents. It may be concluded, therefore, that ferrochelatase-inhibitory activity is the key factor that determines porphyrin-inducing activity of DDC analogues in chick embryo liver cells and that lipophilicity is of less importance.

Our next studies were directed to determine the *in vitro* effects of DDC analogues on hepatic microsomal cytochrome P-450 from chick embryo. All of the analogues investigated (**III–VIII**) caused cytochrome P-450 destruction which was paralleled by the destruction of the heme moiety of cytochrome P-450 (Table 3). The dose of DDC analogues used in these experiments was 125 μg/ml. A series of doses of 4-ethyl DDC (1.67–125 μg/ml) was explored for destructive effects. A dose-response relationship was observed and significant destruction of cytochrome P-450 was observed even at the lowest concentration (1.67 μg/ml). Destruction was anticipated with analogues **III, IV, V,** and **VII** in view of the fact that they caused *N*-alkylprotoporphyrin formation (Table 2) and reduction of ferrochelatase activity (Fig. 2). The destruction of cytochrome P-450 paralleled by loss of heme with the 4-chloromethyl analogue (**VII**) accords with the idea that the ferrochelatase inhibition observed (Fig. 2) may be due to formation of a labile *N*-alkylprotoporphyrin IX moiety derived from the heme moiety of cytochrome P-450. The destruction of cytochrome P-450 observed with the 4-isobutyl analogue (**VI**) accords with results reported with rat hepatic microsomes obtained from phenobarbital-pretreated rats (8). The loss of heme found (Table 3)

accords with the previous finding of an *N*-alkylprotoporphyrin IX (8).

Ortiz de Montellano *et al.* (30) have suggested the following model for the binding of porphyrins in the ferrochelatase active site. The porphyrin is drawn into the proposed active site by hydrogen bonding or electrostatic interactions between its carboxylic acid groups and residues in the active site. The front half of the molecule, including pyrrole rings C and D, is bound in a sterically constrained region that tolerates little substitution on the pyrrole nitrogens. The back half of the molecule, however, remains in a relatively open region of the active site and thus is subject to much looser steric limits on the size of *N*-alkyl groups attached to pyrrole rings A and B.

On the basis of the above model, the inability of the 4-isobutyl analogue (VI) to reduce ferrochelatase activity may be due to either: (a) the fact that the *N*-isobutyl group is located exclusively on either or both of the C and D rings, or (b) the fact that a branched chain alkyl group is sterically incompatible with the active site of ferrochelatase even when located on either or both of rings A and B. On the basis of the above model it would appear that the ferrochelatase-inhibitory activity of the 4-butyl (III), 4-phentyl (IV), and 4-hexyl analogues (V) must be due to the formation of the corresponding *N*-alkylporphyrins containing *N*-alkyl substituents on either or both the A and B rings. In order to resolve these questions, the *N*-alkylporphyrin regioisomers derived from the analogues must be separated, characterized, and tested for ferrochelatase-inhibitory activity.

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