MOLECULAR PHARMACOLOGY, 20:395–403

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

SUSAN P. C. COLE,[1] RALPH ALLEN WHITNEY,[2] AND GERALD S. MARKS[1]

Departments of Pharmacology and Chemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

Received December 29, 1980; Accepted April 20, 1981

SUMMARY

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).

A series of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) was investigated for ferrochelatase-inhibitory and porphyrin-inducing activities in chick embryo liver cell cultures. It was found that, in order to inhibit ferrochelatase maximally, a DDC analogue must be a dihydropyridine with ester groups in positions 3 and 5, and an alkyl substituent in position 4. The structural requirements for porphyrin-inducing activity were less stringent. The pattern of porphyrin accumulation produced in response to DDC and its analogues was investigated using high-performance liquid chromatography. Protoporphyrin was found to be the major porphyrin to accumulate in response to dihydropyridine analogues that inhibited ferrochelatase. Uro- and heptacarboxylic porphyrins were the major porphyrins to accumulate in response to all of the pyridine analogues. A pattern of porphyrin accumulation very similar to that produced by the pyridine analogues was observed with those dihydropyridine analogues which did not inhibit ferrochelatase, suggesting that these dihydropyridines are readily converted to pyridines in the chick embryo liver cell culture. It is clear that some analogues of DDC induce porphyrin accumulation by a mechanism other than through ferrochelatase inhibition.

INTRODUCTION

One mechanism by which porphyrinogenic compounds can disrupt the regulation of the heme biosynthetic pathway is through inhibition of ferrochelatase (EC 4.99.1.1), the terminal enzyme of the pathway. Inhibition of ferrochelatase should result in decreased heme formation, diminished heme-mediated feedback repression of the rate-limiting enzyme ALA[3] synthetase (EC 2.3.1.37), followed by increased ALA synthetase activity leading to an accumulation of porphyrins (1). Although porphyrin accumulation can be induced in experimental animals by numerous chemicals of diverse structures, only DDC (Compound I) and griseofulvin have been reported to inhibit ferrochelatase in rodents (2, 3). DDC has also been shown to be a potent inhibitor of ferrochelatase in the 17-day-old chick embryo (4–6) and, more recently, in the chick embryo liver cell cultures (7). In contrast, griseofulvin does not inhibit ferrochelatase in these avian systems (7). Thus DDC remains unique among porphyrin-inducing chemicals in its ability to inhibit ferrochelatase in the 17-day-old chick embryo and chick embryo liver cell culture. Therefore, it was of interest to determine the structural features of DDC required for ferrochelatase inhibition. A preliminary study in the intact 17-day-old chick embryo has been presented elsewhere (8). This study has now been extended to chick embryo liver cell culture since it had previously been noted that, although a DDC analogue may be unable to cause porphyrin accumulation in the intact embryo, it may still have marked activity in the cell culture system (9). For example, even though Ox-DDC (Compound XV) has no porphyrinogenic effect *in vivo*, it causes marked porphyrin accumulation in the cell culture system (10).

This work was supported by the Medical Research Council of Canada and the Natural Sciences and Engineering Research Council.

[1] Department of Pharmacology.

[2] Department of Chemistry.

[3] The abbreviations used are: ALA, δ-aminolevulinic acid; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Compound I); Ox-DDC, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (Compound XV); desmethyl-DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Compound II); BNPP, bis[*p*-nitrophenyl]phosphate; HCB, hexachlorobenzene.

0026-895X/81/020395-09$2.00/0

The objective of this study was to determine the structural features required in DDC for inhibition of ferrochelatase in chick embryo liver cell cultures and to compare them with the structural features required for porphyrin accumulation.

Recently Tephly *et al.* (11) and De Matteis and coworkers (12) have shown that, when DDC is injected into rodents, it causes accumulation of green pigments, apparently derived from heme breakdown, which strongly inhibit ferrochelatase. They have shown spectrophotometrically that the inhibitory pigments closely resemble *N*-monomethylporphyrins and have concluded that administration of DDC gives rise to an *N*-alkyl porphyrin which in turn inhibits ferrochelatase. Their conclusion is supported by the finding that *N*-methylmesoporphyrin and *N*-methylprotoporphyrin inhibit mouse liver ferrochelatase *in vitro* (13). Examination of the ferrochelatase-inhibitory properties of selected DDC analogues (Tables 1 and 2) should aid in the analysis of the structural requirements for ferrochelatase inhibition and thus may provide some insight into the mechanism by which DDC causes accumulation of an inhibitory porphyrin.

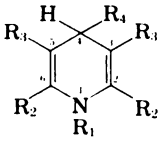
METHODS

Source of compounds. DDC (Compound I), 3,5-pyridinedicarboxylic acid, 3,5-diethoxycarbonyl-2,6-dimethylpyridine (Compound XVI), and desmethyl-DDC (Compound II) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisc.). BNPP was obtained from the Sigma Chemical Company (St. Louis, Mo.). Porphyrins and porphyrin methyl esters were purchased from Porphyrin Products (Logan, Utah).

The identity and purity of the DDC analogues used in this study were verified by melting point determination, NMR spectral analysis, elemental analysis, and thin-layer chromatography. Melting points were determined on a Fisher Johns or a Thomas-Hoover apparatus and

TABLE 1

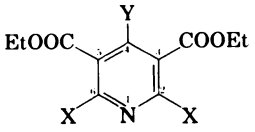
Structure of the dihydropyridine analogues of DDC



Analogue	R_1	R_2	R_3	R_4
I	H	CH_3	$COOC_2H_5$	CH_3
II	H	CH_3	$COOC_2H_5$	H
III	H	CH_3	$COOC_2H_5$	C_2H_5
IV	H	CH_3	$COOC_2H_5$	C_3H_7
V	H	CH_3	$COOC_2H_5$	C_6H_5
VI	H	CH_3	CN	CH_3
VII	H	CH_3	$COCH_3$	CH_3
VIII	H	CH_3	$COOCH_2C_6H_5$	CH_3
IX	H	CH_3	$COOC(CH_3)_3$	CH_3
X	H	CH_3	$COOC(CH_3)_3$	H
XI	H	H	$COOC_2H_5$	CH_3
XII	CH_3	CH_3	$COOC_2H_5$	CH_3
XIII	CH_3	CH_3	$COOC_2H_5$	H
XIV	C_2H_5	CH_3	$COOC_2H_5$	CH_3

TABLE 2

Structure of the pyridine analogues of DDC



Analogue	*X*	*Y*
XV	CH_3	CH_3
XVI	CH_3	H
XVII	H	H

are uncorrected. A Brucker HX60 or Varian EM360 instrument was used to record NMR spectra. All compounds were analyzed for carbon, hydrogen, and nitrogen by Guelph Chemical Laboratories, Ltd. (Guelph, Canada), and the results were within ±0.3% of the theoretical value. UV spectra were recorded on a Unicam SP800 or Cary 219 spectrophotometer.

Many of the dihydropyridines have been previously synthesized in our laboratory (10, 14) by a general method described for this class of compounds by Loev and Snader (15). Ox-DDC (Compound XV) was prepared by oxidation of DDC (Compound I) with a mixture of glacial acetic acid and sodium nitrite by the method of Loev and Snader (15). 3,5-Diethoxycarbonylpyridine (Compound XVII) was prepared by esterification of 3,5-pyridinedicarboxylic acid with HCl gas in ethanol, according to the general procedure described by Weygand (16). 3,5-Diethoxycarbonyl-1,4-dihydro-4-methylpyridine (Compound XI) was prepared by reaction of methylmagnesium iodide with 3,5-diethoxycarbonylpyridine (Compound XVII) as described by Brignell *et al.*(17). The *N*-methyl analogues (Compounds XII and XIII) were synthesized by treatment of their corresponding dihydropyridines (Compounds I and II) with dimsylsodium in dimethyl sulphoxide followed by addition of methyl iodide as described by Brignell *et al.* (17).

The *N*-ethyl analogue (Compound XIV) (3,5-diethoxycarbonyl-1,4-dihydro-1-ethyl-2,4,6-trimethylpyridine) was prepared by using the general procedure of Brignell *et al.* (17). To a solution of dimsylsodium [from sodium hydride (1.44 g, 50% dispersion in oil, 30 mmoles) and dimethyl sulfoxide (20 ml)] under nitrogen was added DDC (Compound I) (2.0 g, 7.1 mmoles) in dimethyl sulfoxide (20 ml). The reaction mixture was stirred for 5 min at room temperature, after which ethyl bromide (2.8 ml, 4.08 g, 37.4 mmoles) was added. Stirring was continued for an additional 16 hr and then the mixture was poured into water (400 ml) and extracted with methylene chloride (200 ml). The methylene chloride layer was washed with HCl (10%), followed by sodium bicarbonate (5%). After drying, the solution was filtered and the solvent was removed under reduced pressure. Recrystallization of the residue from petroleum ether (30–60°) afforded a crystalline product, m.p. 69.5–70° (0.41 g, 1.4 mmoles; yield 20%); NMR ($CDCl_3$), 4.18 δ (OCH_2, 4H quartet, $J = 7$ Hz), 3.69 δ (NCH_2, 2H, quartet, $J = 7$ Hz), 2.39 δ (2,6-CH_3, 6H, singlet), 1.29 δ (CH_3, 6H, triplet, $J = 7$ Hz), 1.15 δ (CH_3, 3H, triplet, $J = 7$ Hz), 0.89 δ (CH_3,

3H, doublet, J = 6.5 Hz); UV (ethanol), λ_{max} 345, 261, 233, and 195 nm (ϵ 14,600, 18,400, 27,050, and 12,725).

$C_{16}H_{25}NO_4$

Calculated: C 65.1, H 8.5, N 4.8

Found: C 64.8, H 8.7, N 4.9

Determination of ALA synthetase activity and ferrochelatase activity. The details of the cell culture technique have been described previously (18). The cells were cultured in serum-free Waymouth MD 705/1 medium supplemented with penicillin G sodium, streptomycin sulfate, insulin, and L-thyroxine sodium pentahydrate. After an initial incubation period of 24 hr, the medium was discarded and replaced with fresh medium. For the assay of ferrochelatase and ALA synthetase activities, the cells were maintained in 10-cm diameter disposable plastic dishes containing 15 ml of the medium. Chemicals were dissolved in 95% ethanol for addition to the dishes (maximal total volume added: 30 μl). ALA synthetase activity was assayed 12 hr later and ferrochelatase activity was assayed 24 hr later, unless otherwise indicated. ALA synthetase activity was measured as described previously (19). For the determination of ferrochelatase activity, 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 were added to each dish. The cells from two similarly treated dishes were pooled and centrifuged (500 × g) for 5 min. The cell pellet was washed once and then suspended in 2.0 ml of ice-cold 0.02 M Tris-HCl buffer, pH 8.2. The cell suspension was homogenized by using a Polytron homogenizer. Ferrochelatase activity of a 0.8-ml aliquot of the homogenate was measured as described previously (6) by a modification of the pyridine hemochromogen method (20), using mesoporphyrin and iron as substrates.

Determination of total porphyrins and porphyrin profiles. For the determination of total porphyrins and porphyrin patterns, chick embryo liver cells were maintained in 6-cm diameter dishes containing 5 ml of the medium. Drugs were dissolved in 95% ethanol for addition to the dishes (maximal total volume: 15 μl). Total porphyrins were assayed 24 hr after addition of the drugs, as previously described (21). Porphyrin patterns were determined by the second-derivative high-performance liquid chromatographic method of Zelt *et al.* (22). Since protoporphyrin is particularly labile to mineral acids (23), a known amount of protoporphyrin [E_{mM}^{408nm} (2.7 N HCl) = 262] was added to four control dishes to correct for losses due to the esterification and extraction procedures. Recovery of the other porphyrins was consistently greater than 90% (24); thus internal standards were not required. The contents of each dish were lyophilized and esterified at −15° with 5% sulfuric acid in methanol for 24 hr. The methyl esters were extracted into chloroform, washed with 5% sodium bicarbonate and with water, dried over anhydrous sodium sulfate, and then evaporated to dryness under nitrogen at 37°. The residue was taken up in mobile phase (hexane/ethyl acetate/methanol) (60/37/3; v/v/v) (200 μl), and a 10-μl aliquot was injected onto the column. Results were calculated as picomoles of porphyrin per milligram of protein and are expressed as percentage of total porphyrins.

Protein determinations. Protein was assayed by the method of Lowry *et al.* (25).

RESULTS

The percentage inhibition of ferrochelatase in chick embryo liver cell culture 24 hr after the addition of increasing amounts of DDC (Compound I) is shown in Fig. 1. Inhibition of activity was observed at doses of DDC as low as 0.001 μg/ml of medium, whereas maximal inhibition was achieved at 0.03 μg/ml of medium. Ferrochelatase inhibition (approximately 40%) was observed as early as 1 hr after addition of the drug (5 μg/ml of medium) and was maximal at about 6 hr (results not shown). The effect of increasing doses of DDC on ALA synthetase activity 12 hr after addition of the drug is shown in Fig. 1. No significant increase in activity was detected below 1 μg of DDC per milliliter of medium.

The effects of DDC (Compound I) and 3,5-diethoxycarbonyl-1,4-dihydro-4-methylpyridine (Compound XI) on ferrochelatase activity and porphyrin accumulation are shown in Fig. 2. DDC maximally inhibited ferrochelatase at 0.03 μg of DDC per milliliter of medium; a significant increase in porphyrin levels was not observed until a dose of 1 μg of DDC per milliliter of medium (Fig. 2a) was administered. Maximal inhibition of ferrochelatase by Compound XI was not achieved until 1.0 μg of drug per milliliter of medium (Fig. 2b) was administered. Thus, this analogue is about 30 times less potent than DDC. Porphyrin levels increased significantly at 0.3 μg of drug per milliliter of medium. At higher concentrations of Compound XI (>50 μg/ml of medium), porphyrin levels decreased and the degree of inhibition of ferrochelatase activity diminished.

The effects of the *N*-alkylated DDC Analogues XII

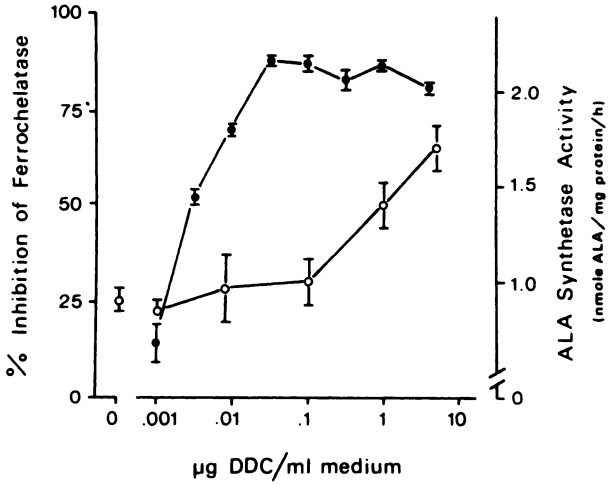


FIG. 1. *ALA synthetase activity at 12 hr* (○) *and percentage inhibition of ferrochelatase activity at 24 hr* (●) *after the administration of increasing doses of DDC to chick embryo liver cell cultures*

The concentration of DDC is shown on a log scale. For ferrochelatase inhibition, each *point* represents the mean of four determinations (± standard error of the mean). For ALA synthetase activity, each *point* represents the mean of five determinations (± standard error of the mean).

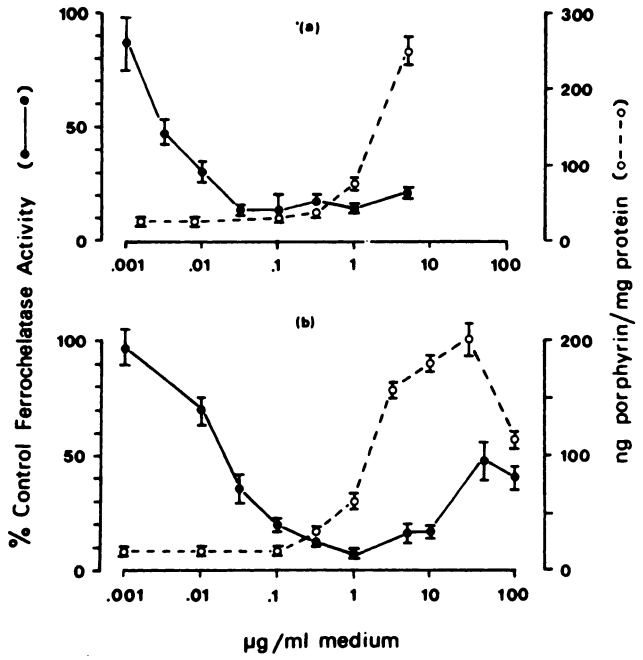


FIG. 2. *Percentage of control ferrochelatase activity* (●——●) *and porphyrin accumulation* (○- - -○) *in chick embryo liver cell cultures 24 hr after administration of increasing doses of (a) DDC (Compound I) and (b) 3,5-diethoxycarbonyl-1,4-dihydro-4-methylpyridine (Compound XI)*

The drug concentrations are shown on a log scale. For ferrochelatase activity, each *point* represents the mean of four determinations (± standard error of the mean). For porphyrin accumulation, each *point* represents the mean of five determinations (± standard error of the mean).

and XIV are shown in Fig. 3. The *N*-methyl analogue (Compound XII) (Fig. 3a) and the *N*-ethyl analogue (Compound XIV) (Fig. 3b) exerted maximal inhibition of ferrochelatase at 0.1 and 0.03 µg of drug per milliliter of medium, respectively. Thus, these *N*-alkylated analogues are approximately as potent as DDC as inhibitors of ferrochelatase. A significant increase in porphyrin levels induced by these analogues did not occur until amounts of drug greater than 0.3 and 0.1 µg per milliliter of medium, respectively, were added to the dishes. Other dihydropyridine analogues were tested for ferrochelatase-inhibitory activity and porphyrin-inducing activity and the results are shown in Table 3. Marked inhibition of ferrochelatase activity was observed with the 4-ethyl (Compound III), 4-propyl (Compound IV), 3,5-dibenzyl ester (Compound VIII) and 3,5-di-*t*-butyl ester (Compound IX) analogues of DDC. These compounds have previously been shown to cause marked porphyrin induction (10, 14). The 3,5-dicyano analogue (Compound VI) did not significantly inhibit ferrochelatase, nor did it cause porphyrin accumulation (Table 3). The 3,5-diacetyl analogue (Compound VII) produced a 56% inhibition of ferrochelatase activity at 10 µg of drug per milliliter of medium, but no significant inhibition at 1 µg/ml of medium; no porphyrin accumulation was observed at either dose (Table 3). Little or no ferrochelatase inhibition was observed with the 4-phenyl analogue (Compound V) and 3,5-di-*t*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Compound X). However, both compounds had porphyrin-inducing activity. Thus, the 4-phenyl analogue (10 µg/ml of medium) produced accumulation of 398.4 ± 40.6 ng of porphyrin per milligram of protein and 3,5-di-*t*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Compound X) (5 µg/ml of medium) increased porphyrin levels to 182.1 ± 6.0 ng of porphyrin per milligram of protein; control levels were 18.9 ± 1.7 ng of porphyrin per milligram of protein.

The effect of desmethyl-DDC (Compound II) (30 µg/ml of medium) on ferrochelatase activity in the presence of BNPP (10 µg/ml of medium) and in corresponding controls is shown in Fig. 4. Addition of desmethyl-DDC alone caused a slight but insignificant decrease in ferrochelatase activity. On the other hand, BNPP alone reduced ferrochelatase activity to approximately 65% of control levels. Coadministration of desmethyl-DDC and BNPP did not result in any further decrease in activity than that observed with BNPP alone. Similar results (not shown) were found with 3,5-diethoxycarbonyl-1,4-dihydro-1,2,6-trimethylpyridine (Compound XIII) (*N*-methyl-4-desmethyl-DDC) (20 µg/ml of medium) in the presence and absence of BNPP.

The effects of desmethyl-DDC (Compound II) (20 µg/ml of medium) and *N*-methyl-4-desmethyl-DDC (Compound XIII) (20 µg/ml of medium) on porphyrin accumulation in the presence of BNPP (10 µg/ml of medium) and in corresponding controls is shown in Fig. 5. Neither

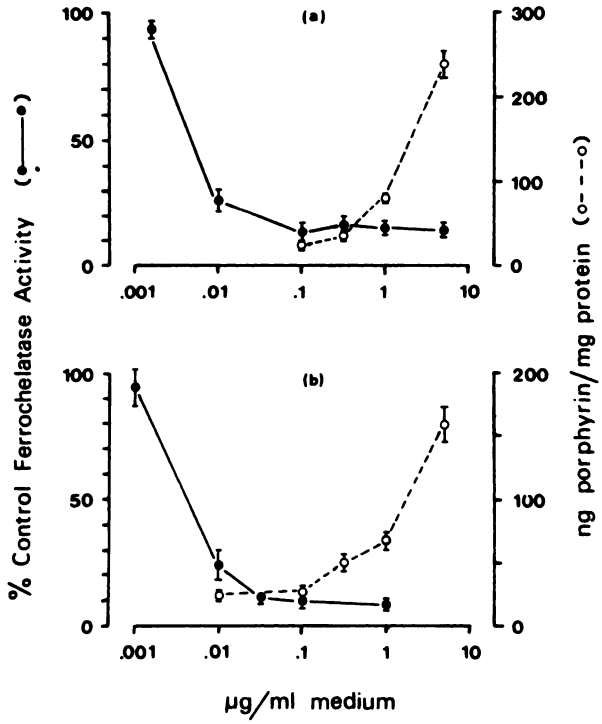


FIG. 3. *Percentage of control ferrochelatase activity* (●——●) *and porphyrin accumulation* (○- - -○) *in chick embryo liver cell cultures 24 hr after administration of increasing doses of (a) N-methyl DDC (Compound XII) and (b) N-ethyl DDC (Compound XIV)*

The drug concentrations are shown on a log scale. For ferrochelatase activity, each *point* represents the mean of four determinations (± standard error of the mean). For porphyrin accumulation, each *point* represents the mean of five determinations (± standard error of the mean).

TABLE 3

Porphyrin-inducing and ferrochelatase-inhibitory properties of dihydropyridine analogues of DDC in chick embryo liver cell culture

Dihydropyridine analogues	Compound no.	Dose	Porphyrin-inducing activity[a]	Ferrochelatase activity[b]
		μg/ml medium		*% control*
DDC	I	1	+	9.2 ± 1.8
desmethyl-DDC	II	30	−	85.5 ± 14.0
4-Ethyl	III	5	+	18.0 ± 1.8
4-Propyl	IV	5	+	11.8 ± 3.1
4-Phenyl	V	5	+	106.1 ± 9.5
3,5-Dicyano	VI	10	−	93.2 ± 13.9
3,5-Diacetyl	VII	1	−	83.9 ± 8.9
		10	−	43.7 ± 8.0
3,5-Dibenzyl ester	VIII	10	+	9.5 ± 1.3
3,5-Di-*t*-butyl ester	IX	5	+	24.7 ± 6.2
3,5-Di-*t*-butyl ester-4-desmethyl	X	5	+	112.6 ± 8.1
3,5-Diethoxycarbonyl-1,4-dihydro-4-methylpyridine	XI	1	+	11.8 ± 2.2
N-methyl-DDC	XII	1	+	14.6 ± 1.3
N-methyl-4-desmethyl-DDC	XIII	20	−	96.0 ± 8.5
N-ethyl-DDC	XIV	1	+	8.5 ± 1.5

[a] Based on results in refs. 10, 14, and 28 and data presented in this paper. + Denotes porphyrin levels significantly greater than control, and − denotes no significant increase over control values.

[b] Values given are means (± standard error of the mean) of four determinations.

BNPP nor desmethyl-DDC (Compound II) increased porphyrin levels above control values (29.9 ± 1.5 ng of porphyrin per milligram of protein) when added to the dishes alone; however, when added together, a marked porphyrin accumulation was observed (128.1 ± 7.3 ng of porphyrin per milligram of protein). In contrast, coadministration of *N*-methyl-4-desmethyl-DDC (Compound XIII) (20 μg/ml of medium) and BNPP (10 μg/ml of medium) resulted in only a slight but significant increase in porphyrin levels (42.8 ± 2.4 ng of porphyrin per milligram of protein).

Several pyridine analogues were tested for ferrochelatase-inhibitory activity and porphyrin-inducing activity and the results are shown in Table 4. Ox-DDC (Compound XV) caused a 35% inhibition of ferrochelatase activity, whereas 3,5-diethoxycarbonylpyridine (Compound XVII) and desmethyl-Ox-DDC (Compound XVI) were without effect. Ox-DDC (Compound XV) (50 μg/ml of medium) caused marked porphyrin accumulation (495.5 ± 17.5 ng of porphyrin per milligram of protein) compared with control values (19.2 ± 0.6 ng of porphyrin per milligram of protein), whereas Compounds XVI and XVII were inactive.

Pyridine analogues lacking a 4-alkyl substituent (desmethyl-Ox-DDC, Compound XVI; 3,5-diethoxycarbon-

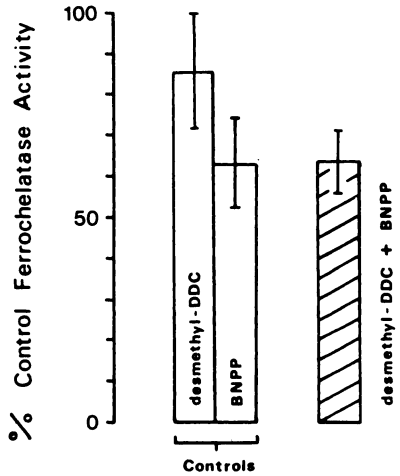


FIG. 4. *Percentage of control ferrochelatase activity in chick embryo liver cell cultures 24 hr after administration of desmethyl-DDC (Compound II) (30 μg/ml of medium) in the presence of BNPP (10 μg/ml of medium) (hatched bar) and in corresponding controls (open bars)*

Each *bar* represents the mean of four determinations (± standard error of the mean).

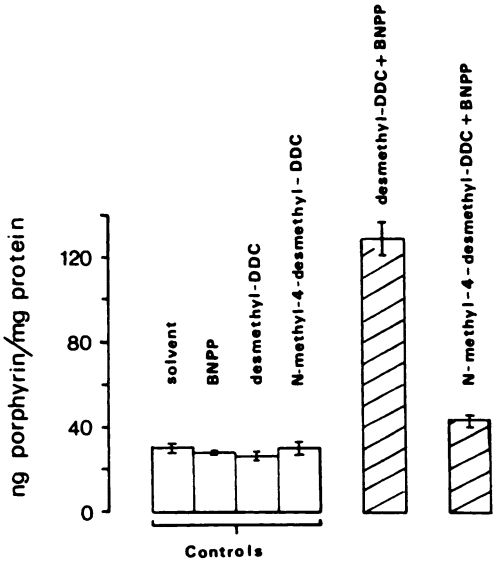


FIG. 5. *Porphyrin accumulation in chick embryo liver cell cultures 24 hr after administration of desmethyl-DDC (Compound II) (20 μg/ml of medium) and N-methyl-4-desmethyl-DDC (Compound XIII) (20 μg/ml of medium) in the presence of BNPP (10 μg/ml of medium) (hatched bars) and in corresponding controls (open bars)*

Each *bar* represents the mean of five determinations (± standard error of the mean).

TABLE 4

Porphyrin-inducing and ferrochelatase-inhibitory properties of pyridine analogues of DDC in chick embryo liver cell culture

Pyridine analogue	Compound no.	Dose	Porphyrin-inducing activity[a]	Ferrochelatase activity[b]
		μg/ml medium		*% control*
Ox-DDC	XV	20	+	64.3 ± 8.2
desmethyl-Ox-DDC	XVI	50	−	99.5 ± 15.4
3,5-Diethoxycarbonylpyridine	XVII	50	−	118.6 ± 8.0

[a] Based on results in refs. 10, 14, and 28 and data presented in this paper. The (+) denotes porphyrin levels significantly greater than control and the (−) denotes no significant increase over control values.

[b] Values given are the mean (± standard error of the mean) of four determinations.

ylpyridine, Compound XVII) were retested for porphyrin-inducing activity and ferrochelatase-inhibitory activity in the presence of BNPP. No inhibition of ferrochelatase activity was observed with either compound (results not shown). On the other hand, porphyrin accumulation in response to both analogues was greatly enhanced by coadministration of BNPP as shown in Fig. 6.

The porphyrin patterns produced by the dihydropyridine Compounds V, IX, X, DDC (Compound I), desmethyl-DDC (Compound II) in the presence of BNPP, and the pyridine desmethyl-Ox-DDC (Compound XVI) in the presence of BNPP, were determined by separation and quantitation of the porphyrins as their methyl esters by high-performance liquid chromatography. The results are shown in Fig. 7. After administration of DDC (Compound I) (Fig. 7a) and Analogue IX (Fig. 7d), protoporphyrin accounted for 77 and 58%, respectively, of the total porphyrins. In the case of DDC, most of the remainder was coproporphyrin (15%), whereas in the case of Analogue IX most of the remainder was composed of equal amounts of copro- and uroporphyrins (14% each). The pattern of porphyrin accumulation after addition of Compound X (Fig. 7e), desmethyl-Ox-DDC (Compound XVI) in the presence of BNPP (Fig. 7c), and desmethyl-DDC (Compound II) in the presence of BNPP (Fig. 7b) was markedly different from DDC and Analogue IX. These 4-unsubstituted analogues all gave a similar pattern in which uroporphyrin was the major porphyrin to accumulate (48–57%), whereas less than 5% was protoporphyrin. Significant amounts of heptacarboxylic (21–31%) and copro (10–17%) porphyrins were also present. The porphyrin pattern produced by the 4-phenyl analogue (Compound V) of DDC is shown in Fig. 7f. In this case, uroporphyrin accounted for 42% of the total porphyrin, whereas equal amounts of heptacarboxylic- and coproporphyrins (21 and 23%, respectively) comprised most of the remainder.

DISCUSSION

In order to establish a baseline for comparison of the DDC analogues, the relationships among the dose of DDC, ferrochelatase inhibition, porphyrin levels, and ALA synthetase activity in chick embryo liver cell cultures were investigated. Although maximal inhibition of ferrochelatase activity was observed at 0.03 μg of DDC per milliliter of medium, no significant increase in ALA synthetase activity (Fig. 1) nor in porphyrin levels (Fig. 2a) was detected below 1 μg of DDC per milliliter of medium. The inhibitory effect of DDC on ferrochelatase activity in chick embryo liver cell culture appears to be dissociated from its ability to induce ALA synthetase activity and porphyrin accumulation. These results correspond to previous findings in the 17-day-old chick embryo (5,6).

To explore the role of the methyl groups at positions 2 and 6 of DDC, the analogue (XI) lacking these substit-

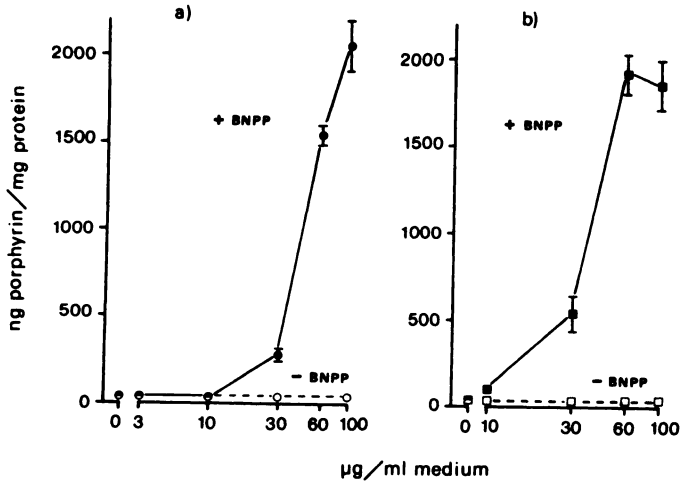


FIG. 6. *Porphyrin accumulation in chick embryo liver cell cultures 24 hr after administration of increasing doses of (a) desmethyl-Ox-DDC (Compound XVI) in the absence (○- - -○) and presence (●——●) of BNPP (10 μg/ml of medium) and (b) 3,5-diethoxycarbonylpyridine (Compound XVII) in the absence (□- - -□) and presence (■——■) of BNPP (10 μg/ml of medium)*

The drug concentrations are shown on a log scale. Each *point* represents the mean of five determinations (± standard error of the mean).

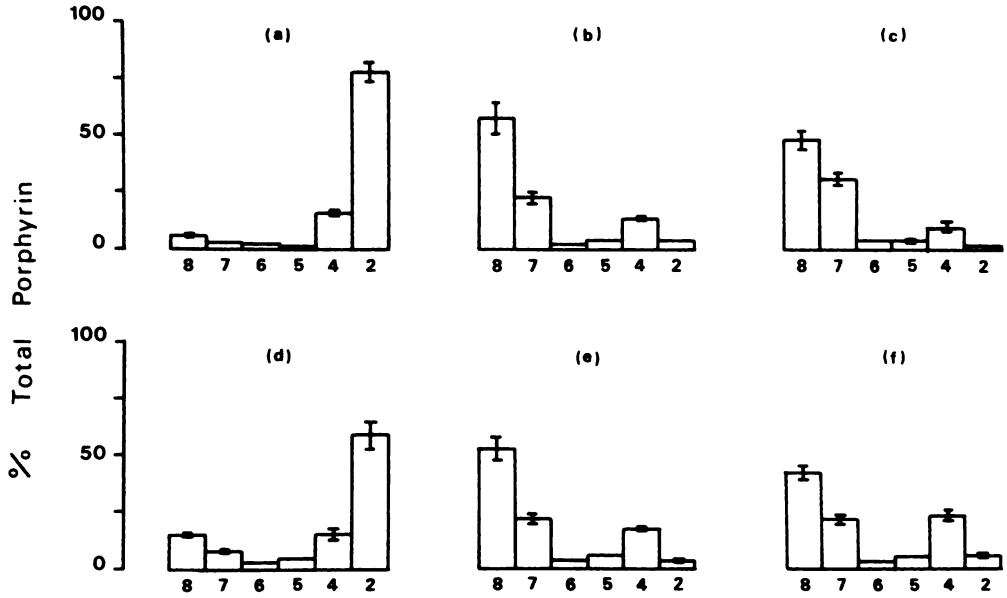


FIG. 7. *Porphyrin patterns produced by (a) DDC (Compound I) (5 μg/ml of medium), (b) desmethyl-DDC (Compound II) (30 μg/ml of medium) in the presence of BNPP (10 μg/ml of medium), (c) desmethyl-Ox-DDC (Compound XVI) (50 μg/ml of medium) in the presence of BNPP (10 μg/ml of medium), (d) 3,5-di-t-butoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Compound IX) (5 μg/ml of medium), (e) 3,5-di-t-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Compound X) (5 μg/ml of medium), and (f) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine (Compound V) (5 μg/ml of medium) in chick embryo liver cell cultures*

Numbers under the *bars* indicate the number of carboxyl groups (*8* = uroporphyrin; *7* = heptacarboxylic porphyrin; *6* = hexacarboxylic porphyrin; *5* = pentacarboxylic porphyrin; *4* = coproporphyrin; *2* = protoporphyrin). Each *bar* represents the mean (± standard error of the mean) of four determinations in one experiment and the results were confirmed in one or two additional experiments. Some variability in levels of coproporphyrin from experiment to experiment was noted. Absolute values of total porphyrins shown here were as follows: (a) 1177.6 pmoles/mg of protein, (b) 515.6 pmoles/mg of protein, (c) 1758.0 pmoles/mg of protein, (d) 2080.3 pmoles/mg of protein, (e) 813.6 pmoles/mg of protein, and (f) 471.2 pmoles/mg of protein. Control levels were 55.6 pmoles/mg of protein.

uents was synthesized (17) and its activity was studied. The potency of Analogue XI as a porphyrin-inducer was comparable to that of DDC at doses of 1 and 5 μg/ml of medium (Fig. 2). Higher doses of DDC were not tested, since they were known from previous studies[4] to result in diminished porphyrin accumulation relative to the 5-μg/ml of medium dose. Analogue XI was found to have approximately 1/30 of the potency of DDC as an inhibitor of ferrochelatase (Fig. 2), although its maximal effect was similar to that of DDC. It was concluded that the methyl groups at positions 2 and 6 of DDC are not essential for porphyrin-inducing or ferrochelatase-inhibitory activity. Higher doses of Analogue XI resulted in diminished porphyrin accumulation and degree of ferrochelatase inhibition relative to the maximal effects found at 30 and 1 μg/ml of medium, respectively.

Our second objective was to determine whether alkylation of the nitrogen atom of DDC would alter the activity of DDC. For this reason, the *N*-methyl analogue (Compound XII) and the *N*-ethyl analogue (Compound XIV) were synthesized (17) and tested. The dose-response curves observed with the *N*-alkylated Analogues XII and XIV (Fig. 3) were comparable to that found with DDC (Fig. 2a). It was concluded that addition of an alkyl group to the nitrogen atom of DDC does not alter the ferrochelatase-inhibitory or porphyrin-inducing properties of DDC.

Our third objective was to determine whether the ethyl ester groups of DDC were essential for ferrochelatase-inhibitory activity and porphyrin-inducing activity. For this reason, analogues were tested in which the 3 and 5 ethoxycarbonyl groups of DDC were replaced with benzyl ester groups (Compound VIII) and *t*-butyl ester groups (Compound IX). It is known from a previous study (14) that these compounds are active porphyrin inducers. In the present study, it was found that these analogues of DDC also retained ferrochelatase-inhibitory activity (Table 3). It was concluded that the ethyl ester groups at positions 3 and 5 of DDC can be replaced by different ester groups without losing porphyrin -inducing or ferrochelatase-inhibitory activities. Analogues in which the 3 and 5 ethoxycarbonyl groups of DDC were replaced with cyano (Compound VI) and acetyl (Compound VII) groups were tested. Neither of these analogues caused porphyrin accumulation, and only the diacetyl analogue (Compound VII) exerted ferrochelatase inhibitory activity (Table 3). However, the potency of the diacetyl analogue as a ferrochelatase inhibitor was about 3000-fold less than that of DDC. Thus, Analogue VII reduced ferrochelatase activity to 44% of control values (Table 3) at a concentration of 10 μg/ml of medium, whereas DDC caused a similar inhibition at 0.003 μg/ml of medium (Fig. 1). It was therefore concluded that ester groups in positions 3 and 5 of DDC were required for prophyrin-inducing activity and for maximal ferrochelatase-inhibitory activity.

To determine whether the dihydropyridine ring structure of DDC was essential for ferrochelatase-inhibitory activity, pyridine analogues of DDC were tested. It is known from previous studies (10) that the pyridine ana-

[4] G. S. Marks, unpublished observations.

logue of DDC, Ox-DDC (Compound XV), has porphyrin-inducing activity in chick embryo liver cell culture, an observation which was confirmed in this study. Despite its ability to cause considerable porphyrin accumulation, the potency of Ox-DDC (Compound XV) as a ferrochelatase inhibitor was slight compared with that of DDC. Thus, Ox-DDC (Compound XV) (20 μg/ml of medium) produced a 35% inhibition of ferrochelatase activity (Table 4) in contrast to DDC, which produced a similar inhibition at a concentration of 0.002 μg/ml of medium (Fig. 1). Pyridine Analogues XVI and XVII were inactive both as porphyrin inducers and ferrochelatase inhibitors (Table 4). Since these compounds lacked a 4-alkyl substituent thought to be necessary for protection from inactivation by esterases (26), it was anticipated that coadministration of the carboxylesterase inhibitor BNPP with these analogues would render them active. This was found to be the case for porphyrin-inducing activity (Fig. 6); however, the compounds remained inactive as ferrochelatase inhibitors (results not shown). From these results, it was concluded that the dihydropyridine ring was not necessary for porphyrin induction, but was necessary for maximal ferrochelatase inhibition.

Our final objective was to investigate the importance of the 4-alkyl substituent of DDC. For this reason, analogues in which the 4-methyl group of DDC was replaced with an ethyl group (Compound III), a propyl group (Compound IV), a phenyl group (Compound V), and a hydrogen atom (desmethyl-DDC) (Compound II) were examined. It is known from previous studies (10, 14) that the 4-ethyl and 4-propyl analogues are potent porphyrin inducers. In this study, these analogues have been shown to have ferrochelatase-inhibitory activity (Table 3). The 4-phenyl analogue of DDC (Compound V) has been previously reported to induce porphyrin accumulation (27), and its activity was confirmed in this study. However, in contrast to the 4-alkyl Analogues III and IV, the 4-phenyl analogue (Compound V) was completely devoid of ferrochelatase-inhibitory activity (Table 3). Finally, replacement of the 4-methyl substituent with a 4-H atom (desmethyl-DDC) (Compound II) resulted in a compound which had insignificant ferrochelatase-inhibitory activity (Fig. 4) and no porphyrin-inducing activity (Fig. 5) (10). The inactivity of this analogue as a porphyrin-inducing drug has previously been attributed to its ready hydrolysis and inactivation by hepatic esterases (26), since removal of the 4-methyl group would reduce the steric hindrance of the molecule to hydrolysis of the ester groups and yield the inactive diacid. For this reason, desmethyl-DDC (Compound II) was retested in the presence of the carboxylesterase inhibitor, BNPP. As expected, porphyrin accumulation was greatly enhanced (Fig. 5). Surprisingly, coadministration of BNPP and desmethyl-DDC (Compound II) did not result in inhibition of ferrochelatase any greater than that observed with BNPP alone (Fig. 4). These results indicated that the 4-methyl substituent of DDC has a role other than just to protect the ester groups from hydrolysis. It was concluded that the 4-alkyl substituent of DDC was an absolute requirement for ferrochelatase inhibition, although it was not necessary for porphyrin induction. The moderate inhibitory effect exerted by BNPP alone (Fig. 4) on ferrochelatase was not anticipated. Although BNPP is an inhibitor of a variety of esterases and amidases, it may have additional activities that remain to be clarified.

To test the validity of the above conclusion, Analogue X, in which the 4-methyl group of DDC was replaced by a 4-H atom and the ethyl ester groups by *t*-butyl ester groups, was tested for activity. Although it appears that the bulky *t*-butyl ester groups rendered Analogue X resistant to enzymatic hydrolysis and inactivation (14), thus enabling the compound to cause porphyrin induction, this analogue caused little inhibition of ferrochelatase activity (Table 3). On the other hand, the corresponding di-*t*-butyl ester analogue with a 4-methyl substituent (Compound IX) had both porphyrin-inducing and ferrochelatase-inhibitory activities.

In summary, we have shown that, in order to inhibit ferrochelatase maximally in chick embryo liver cell culture, a DDC analogue must have all three of the following structural components: (a) a 1,4-dihydropyridine ring structure, (b) ester groups in positions 3 and 5, and (c) an alkyl substituent in position 4. The structural requirements for porphyrin-inducing activity are less stringent. Thus, pyridine analogues are potent inducers, and analogues lacking a 4-alkyl substituent are also active provided that the ester groups are protected from hydrolysis either sterically or by coadministration of an esterase inhibitor.

Since some dihydropyridine analogues (e.g., Compounds V, X, and II in the presence of BNPP) caused porphyrin accumulation despite their inability to inhibit ferrochelatase, it was of interest to determine the nature of the porphyrins produced. For comparison, the porphyrin patterns produced by DDC (Compound I), desmethyl-Ox-DDC (Compound XVI) in the presence of BNPP and by the di-*t*-butyl ester analogue (Compound IX) were also determined (Fig. 7). DDC (Compound I) (Fig. 7a) and the *t*-butyl ester analogue (Compound IX) (Fig. 7d) produced very similar patterns; the major porphyrin which accumulated was protoporphyrin, a result consistent with their ability to inhibit ferrochelatase. On the other hand, protoporphyrin accounted for less than 5% of the porphyrins induced by the remaining analogues (Fig. 7), which is in accordance with their failure to inhibit ferrochelatase.

It is noteworthy that the porphyrin patterns produced by the 4-unsubstituted dihydropyridine analogues, 3,5-di-*t*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Compound X) (Fig. 7e) and desmethyl-DDC (Compound II) in the presence of BNPP (Fig. 7b), are strikingly similar. Moreover, they are very similar to that produced by desmethyl-Ox-DDC (Compound XVI) in the presence of BNPP (Fig. 7c) and other pyridines [Compound XVII in the presence of BNPP, and Ox-DDC (Compound XV)] (results not shown). Since the 4-unsubstituted dihydropyridines produce a porphyrin pattern which is the same as that observed with the pyridines, it may be assumed that these dihydropyridines are converted to pyridines in the chick embryo liver cell cultures. In this way, it is possible to explain why the pattern observed with the dihydropyridine desmethyl-DDC (Compound II) (Fig. 7b) is the same as that seen with the pyridine desmethyl-Ox-DDC (Compound XVI) (Fig. 7c) and different from that observed with DDC (Compound I) (Fig. 7a). This interpretation is also consistent

with the comparative rates of oxidation of 4-substituted and 4-unsubstituted dihydropyridines by chemical, polarographic, and fermentative methods (28). It seems that, regardless of the oxidation mechanism, 4-unsubstituted dihydropyridines are much more readily oxidized to pyridines than the corresponding 4-substituted compounds.

The proposed conversion of the 4-unsubstituted dihydropyridines to pyridines also provides an explanation for the failure of *N*-methyl-4-desmethyl-DDC (Compound XIII) coadministered with BNPP to induce porphyrin accumulation as was observed with desmethyl-DDC (Compound II) and BNPP (Fig. 5). The presence of the methyl group on the nitrogen atom of Analogue XIII would prevent oxidation to the corresponding pyridine; instead, a pyridinium ion would be formed. As a charged molecule, this compound would be of low lipophilicity and unlikely to cause porphyrin induction (9).

The porphyrin pattern produced by the 4-phenyl analogue (Compound V) (Fig. 7f) is similar to that observed with the pyridines and the 4-unsubstituted dihydropyridines. The failure of the 4-phenyl analogue (Compound V) to inhibit ferrochelatase indicates that the 4-alkyl substituent of DDC plays a role in addition to impeding ester group hydrolysis and oxidation of the dihydropyridine ring. It is possible that it is the source of the alkyl substituent of the *N*-alkylated porphyrin, the direct inhibitor of ferrochelatase formed in response to DDC administration (11–13).

In this investigation, we have shown that the pyridine analogues and some of the dihydropyridine analogues of DDC induce porphyrin accumulation by a mechanism other than through inhibition of ferrochelatase. The main porphyrins which accumulate are uro- and heptacarboxylic porphyrins (at least 63%), a pattern similar to that observed in rat livers after chronic administration of HCB (29). Elder *et al.* (30) have shown that, in rats, HCB inhibits hepatic uroporphyrinogen decarboxylase (EC 4.1.1.37), the enzyme which catalyzes the stepwise decarboxylation of uroporphyrinogen III to coproporphyrinogen III. Thus, the porphyrin pattern observed is thought to result from the HCB-mediated inhibition of this enzyme. In view of these observations, it would be of interest to examine the effects of the aforementioned analogues of DDC on uroporphyrinogen decarboxylase activity in chick embryo liver cell culture.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Scott Follows, Ms. Frances Taylor, and Ms. Anne Weary for their able technical assistance. We are grateful to Ms. Debbie Browne for her help in the preparation of the manuscript.

REFERENCES

1. De Matteis, F., G. Abritti, and A. H. Gibbs. Decreased liver activity of porphyrin-metal chelatase in hepatic porphyria caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine. *Biochem. J.* **134**:717–727 (1973).
2. Onisawa, J., and R. F. Labbe. Effects of diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate on the metabolism of porphyrins and iron. *J. Biol. Chem.* **238**:724–727 (1963).
3. De Matteis, F., and A. H. Gibbs. Stimulation of the pathway of porphyrin biosynthesis in the liver of rats and mice by griseofulvin, 3,5-diethoxycarbonyl-1,4-dihydrocollidine and related drugs: evidence for two basically different mechanisms. *Biochem. J.* **146**:285–287 (1975).
4. Anderson, K. E. Effects of antihypertensive drugs on hepatic heme biosynthesis and evaluation of ferrochelatase inhibitors to simplify testing of drugs for heme pathway induction. *Biochim. Biophys. Acta* **543**:313–327 (1978).
5. Rifkind, A. B. Maintenance of microsomal hemoprotein concentration following inhibition of ferrochelatase activity by 3,5-diethoxycarbonyl-1,4-dihydrocollidine in chick embryo liver. *J. Biol. Chem.* **254**:4636–4644 (1979).
6. 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).
7. Cole, S. P. C., D. T. Zelt, and G. S. Marks. Comparison of the effects of griseofulvin and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine on ferrochelatase activity in chick embryo liver. *Mol. Pharmacol.* **19**:477–480 (1981).
8. Cole, S. P. C., and G. S. Marks. Structural requirements in dihydropyridines for ferrochelatase inhibition and δ-aminolevulinic acid synthetase induction. *Int. J. Biochem.* **12**:989–992 (1980).
9. Marks, G. S. The effect of chemicals on hepatic heme biosynthesis, in *Handbook of Experimental Pharmacology* (F. De Matteis and W. N. Aldridge, eds.), Vol. 44. Springer, Berlin (1978).
10. Marks, G. S., E. G. Hunter, U. K. Terner, and D. Schneck. Studies of the relationship between chemical structure and porphyria-inducing activity. *Biochem. Pharmacol.* **14**:1077–1084 (1965).
11. 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. *Biochem. J.* **180**:241–244 (1979).
12. De Matteis, F., A. H. Gibbs, and T. R. Tephly. Inhibition of protohaem ferrolyase in experimental porphyria. *Biochem. J.* **188**:145–152 (1980).
13. De Matteis, F., A. H. Gibbs, A. H. Jackson, and S. Weerasinghe. Conversion of liver haem into *N*-substituted porphyrins or green pigments. *FEBS Lett.* **119**:109–112 (1980).
14. Roomi, M. W. Porphyrin-inducing activity of a series of pyridine and dihydropyridine compounds. Investigation in a cell culture system. *J. Med. Chem.* **18**:457–460 (1975).
15. Loev, B., and K. M. Snader. The Hantzsch reaction. I. Oxidative dealkylation of certain dihydropyridines. *J. Org. Chem.* **30**:1914–1916 (1965).
16. Weygand, C., in *Weygand/Hilgetag, Preparative Organic Chemistry* (G. Hilgetag and A. Martini, eds.), John Wiley & Sons, Inc., New York, 370 (1972).
17. Brignell, P. J., U. Eisner, and P. G. Farrell. Substituent effects in the ultraviolet spectra of 1,4-dihydropyridines. *J. Chem. Soc. (B)* 1083–1089 (1966).
18. Morgan, R. O., P. W. F. Fischer, J. K. Stephens, and G. S. Marks. Thyroid enhancement of drug-induced porphyrin biosynthesis in chick embryo liver cells maintained in serum-free Waymouth medium. *Biochem. Pharmacol.* **25**:2609–2612 (1976).
19. Tyrrell, D. L. J., and G. S. Marks. Drug-induced porphyrin biosynthesis. V. Effect of protohemin on the transcriptional and post-transcriptional phases of δ-aminolevulinic acid synthetase induction. *Biochem. Pharmacol.* **21**:2077–2093 (1972).
20. Porra, R. J., K. S. Vitols, R. F. Labbe, and N. A. Newton. Studies on ferrochelatase. The effects of thiols and other factors on the determination of activity. *Biochem. J.* **104**:321–327 (1967).
21. Granick, S. The induction *in vitro* of the synthesis of δ-aminolevulinic acid synthetase in chemical porphyria: a response to certain drugs, sex hormones and foreign chemicals. *J. Biol. Chem.* **241**:1359–1375 (1966).
22. Zelt, D. T., J. A. Owen, and G. S. Marks. Second-derivative high-performance liquid chromatographic-fluorometric detection of porphyrins in chick embryo liver cell culture medium. *J. Chromatogr.* **189**:209–216 (1980).
23. Fuhrop, J.-H., and K. M. Smith. Laboratory methods, in *Porphyrins and Metalloporphyrins* (K. M. Smith, ed.). Elsevier, Amsterdam, 835 (1975).
24. Zelt, D. T. (1980). Interactions of chemicals with the liver heme biosynthetic pathway with special reference to polychlorinated biphenyls. Master of Science thesis, Queen's University, Kingston, Ontario, Canada.
25. 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).
26. Schneck, D. W., and G. S. Marks. Studies of the relationship between chemical structure and porphyria-inducing activity. V. Hydrolysis of amides and esters by chick embryo liver amidases and esterases. *Biochem. Pharmacol.* **21**:2509–2518 (1972).
27. Schneck, D. W., W. J. Racz, G. H. Hirsch, G. L. Bubbar, and G. S. Marks. Studies of the relationship between chemical structure and porphyria-inducing activity. IV. Investigations in a cell culture system. *Biochem. Pharmacol.* **17**:1385–1399 (1968).
28. Uldrikis, Y. R., A. O. Kumerova, and G. Y. Dubur. Oxidation of 1,4-dihydropyridines. II. Reactivity during the oxidation of chloranil. *Khim. Geterosiklich. Soedin.* **5**:691–694 (1973).
29. Elder, G. H. Porphyria caused by hexachlorobenzene and other polyhalogenated aromatic hydrocarbons, in *Handbook of Experimental Pharmacology* (F. DeMatteis and W. N. Aldridge, eds.), Vol. 44. Springer, Berlin (1978).
30. Elder, G. H., J. O. Evans, and S. A. Matlin. The effect of the porphyrogenic compound, hexachlorobenzene, on the activity of hepatic uroporphyrinogen decarboxylase in the rat. *Clin. Sci. Mol. Med.* **51**:71–80 (1976).

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