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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

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SUMMARY

The ferrochelatase-reducing 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. A group of DDC analogues was found in which an inability to reduce ferrochelatase activity corresponded with an inability to cause cytochrome P-450 and heme destruction. In a second group of DDC analogues, the ability to reduce ferrochelatase activity corresponded with the ability to cause cytochrome P-450 and heme destruction. These observations support the idea that the protoporphyrin IX moiety of *N*-alkylprotoporphyrin IX originates from the heme moiety of cytochrome P-450. A third group of DDC analogues caused cytochrome P-450 and heme destruction despite an inability to reduce ferrochelatase activity. With this third group of DDC analogues, the heme moiety of cytochrome P-450 is likely degraded to products other than *N*-alkylporphyrins. The inability of several lipophilic DDC analogues [4-benzyl, 4-isopropyl, 4-cyclohexyl, 4-(3-cyclohexenyl)] to reduce hepatic ferrochelatase activity may explain their low porphyrinogenicity. The pattern of porphyrin accumulation produced in response to two DDC analogues that did not inhibit ferrochelatase was investigated using high performance liquid chromatography. Coproporphyrin was the major porphyrin to accumulate in response to the 4-isopropyl analogue and uro- and heptacarboxylic acid porphyrins in response to the 4-benzyl analogue. These patterns of porphyrin accumulation are consistent with the inability of these analogues to inhibit ferrochelatase.

INTRODUCTION

The dihydropyridine DDC[1] (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 reduces hepatic ferrochelatase (EC 4.99.1.1) activity when administered to rodents (2, 3) or the 17-day-old chick embryo (4, 5), but does not exert any direct inhibitory effect since the enzyme is not inhibited *in vitro* by this agent. The importance of the 4-methyl substituent for the porphyrinogenic activity of this chemical was demonstrated in guinea pigs (6), the 17-day-old chick embryo (7), and chick embryo liver cell culture (6, 8) by the finding that 4-desmethyl-DDC (IX, Table 1) was devoid of activity. The 4-methyl substituent was also found to be essential for hepatic ferrochelatase-lowering activity by studies in the 17-day-old chick embryo liver (9). In contrast, replacement of the 4-methyl substituent of DDC by a 4-ethyl substituent (II, Table 1) or a 4-propyl substituent (III, Table 1) led to enhancement of porphyrinogenic activity (6) and retention of ferrochelatase-lowering activity (9). Oxidation of DDC to the corresponding pyridine, viz., OX-DDC, resulted in loss of porphyrinogenic activity in the guinea pig (6) and 17-day-old chick embryo (7).

The mechanism by which DDC causes diminished hepatic ferrochelatase activity has been elucidated in recent years. DDC, when administered to rodents, causes the accumulation in the liver of a green porphyrin (10, 11) identified as *N*-MePP (10–12), which functions as a potent inhibitor of ferrochelatase (10, 11, 13). On the basis of the structure-activity relationships of DDC, it was thought that the source of the *N*-methyl substituent of *N*-MePP might be the 4-methyl group of DDC. This idea has been shown to be correct (14, 15). The ferrochelatase-reducing activity of the 4-ethyl analogue of DDC (II, Table 1) was found to be due to the formation of *N*-

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[1] The abbreviations used are: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (compound I); *N*-MePP, *N*-methylprotoporphyrin IX; BNPP, bis-(*p*-nitrophenyl)phosphate; OX-DDC, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine; ALAS, δ-aminolevulinic acid synthetase.

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ethylprotoporphyrin IX, which also possessed marked ferrochelatase-inhibitory activity.

The next question which arose was the source of the protoporphyrin IX moiety of *N*-MePP. DDC decreases cytochrome P-450 levels *in vivo* (16). Recently, it has been shown that the lowered P-450 levels observed result from destruction of pre-existing cytochrome P-450 and that DDC and the 4-ethyl (II, Table 1) and 4-propyl analogues of DDC (III, Table 1) are suicide substrates for cytochrome P-450 (15, 17–19). It was inferred from these observations that the source of the protoporphyrin IX for *N*-MePP is the heme moiety of cytochrome P-450. If this hypothesis is correct, it would follow that those dihydropyridine analogues of DDC that diminish hepatic ferrochelatase activity (through formation of *N*-substituted protoporphyrin IX compounds) would act as suicide substrates of cytochrome P-450. The first objective of this study was to determine whether this was so. A second objective was to determine the relationship between the porphyrinogenic activity of DDC analogues and their ability to reduce ferrochelatase activity in chick embryo liver cell culture.

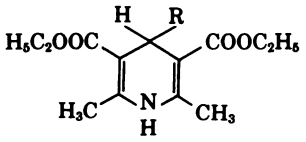
MATERIALS AND METHODS

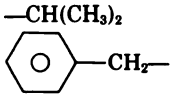
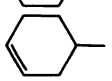
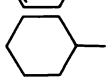
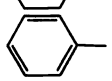
Source of compounds. DDC (I) and its 4-ethyl (II), 4-propyl (III), 4-phenyl (VIII), and 4-desmethyl (IX, Table 1) analogues were obtained as described previously (20). OX-DDC was obtained by oxidation of DDC (I) as described previously (19).

The procedure of Loev and Snader (21) was used to synthesize the following DDC analogues: 4-isopropyl (IV), 4-benzyl (V), 4-cyclohexyl (VII), and 4-(3-cyclohexenyl) (VI, table 1). The identity and purity of the DDC analogues were verified by melting point determination, UV and NMR spectral analysis, and elemental analysis. All compounds were analyzed for carbon, hydrogen, and nitrogen by Guelph Chemical Laboratories, Ltd. (Guelph, Canada) and values obtained were found to be close to the theoretical values. The mass spectra of DDC (I) and its 4-ethyl (II), 4-propyl (III), and 4-isopropyl (IV) analogues were examined and found to be in accord with their structures. BNPP was obtained from Sigma Chemical Co. (St. Louis, MO). Porphyrins and porphyrin methyl esters were purchased from Porphyrin Products (Logan, UT).

TABLE 1

Structure of the dihydropyridine analogues of DDC



Analogue	R
I	CH_3
II	C_2H_5
III	C_3H_7
IV	$-CH(CH_3)_2$
V	
VI	
VII	
VIII	
IX	H

Determination of ferrochelatase activity. The details of the cell culture technique have been described previously (22). 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. The cells were maintained in 10-cm-diameter disposable plastic dishes containing 15 ml of the medium. After an initial incubation period of 24 hr, the medium was discarded and replaced with fresh medium. After a further 24-hr incubation period, chemicals, dissolved in 95% ethanol, were added to the dishes (maximal total volume added, 30 μl). Ferrochelatase activity was assayed 6 hr after addition of DDC and analogues. 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 using a Potter-Elvehjem homogenizer. Ferrochelatase activity of a 0.8-ml aliquot of the homogenate was measured as described previously (5, 20) by a modification of the pyridine hemochromogen method (23) using mesoporphyrin and iron as substrates.

Determination of total porphyrins and porphyrin precursors. 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 (8). Porphyrin patterns were determined by the second derivative high performance liquid chromatographic method of Zelt *et al.* (24). Since protoporphyrin is particularly labile to mineral acids, 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. The recovery of protoporphyrin was approximately 45% (25). 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 with 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/mg of protein and are expressed as percentage of total porphyrins.

Isolation of chick embryo liver microsomes (26). The livers of eight 18-day-old chick embryos were washed by immersion three times in a fresh cold solution prepared by combining 900 ml of 1.15% potassium chloride solution with 100 ml of 0.1 M potassium phosphate buffer, pH 7.4. The livers were homogenized in the cold with 5 volumes of buffered potassium chloride solution, pH 7.4, in a Potter-Elvehjem apparatus. The homogenate was centrifuged (900 × *g*) at 0° for 10 min and the supernatant was removed and centrifuged (9,000 × *g*) at 0° for 20 min. After centrifugation of the supernatant (106,000 × *g*) at 0° for 60 min, the supernatant was discarded and the microsomal pellet was resuspended in buffered potassium chloride solution (16 ml). The microsomal suspension was centrifuged at 106,000 × *g* for 60 min at 0°, the supernatant was discarded, and the microsomes were resuspended in 0.1 M potassium phosphate buffer, pH 7.4 (16 ml). An aliquot (1 ml) of the suspension was used for protein determination.

In vitro cytochrome P-450 and heme destruction. A DDC analogue (500 μg) dissolved in ethanol (20 μl) was added to an Erlenmeyer flask (25 ml) and the ethanol was allowed to evaporate at room temperature. The following additions were made to the Erlenmeyer flask: microsomal suspension (2 ml) and a 2 mM solution of NADPH in 0.1 M phosphate

buffer, pH 7.4, containing EDTA (3.7 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 milligrams 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 baseline was determined by scanning from 500 to 400 nm. Carbon monoxide was run into the sample cuvette for 30 sec and the difference spectrum was recorded from 500 to 400 nm. The quantity of cytochrome P-450 was calculated using the absorbance differences of the peak at 450 nm and the baseline and a molar extinction coefficient of 9.1×10^{-4} (26). The following controls were run: (a) substitution of a 0.1 M phosphate buffer solution containing EDTA (3.7 mM) (2 ml) for the NADPH plus EDTA solution; (b) substitution of ethanol (20 μl) for the DDC analogue solution; (c) substitution of ethanol (20 μl) for the DDC analogue solution and substitution of 0.1 M phosphate buffer solution containing EDTA (3.7 mM) (2 ml) for the NADPH plus EDTA solution.

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 milligrams of sodium dithionite were added to the sample cuvette and 17 μl of 3 mM potassium ferricyanide was 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 (26).

Protein determinations. Protein was assayed by the method of Lowry *et al.* (27).

Statistical analyses. 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 (Table 3) and some of the ferrochelatase data (Table 2). When it was necessary to compare more than two means (i.e., 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 ($p \leq 0.05$).

RESULTS AND DISCUSSION

The reason for the lability of the 4-methyl group of DDC and for the transfer of a C-methyl group to one or more of the four nitrogen atoms of protoporphyrin IX requires clarification. Chemical precedent exists for the loss of a 4-alkyl substituent (21); oxidation of some DDC analogues to the corresponding pyridine proceeds with competitive loss of either the 4-alkyl group (reaction 2, Fig. 1) or the 4-hydrogen atom (reaction 1, Fig. 1). The course of the reaction (dealkylation or proton loss) has been suggested to be determined by the stability of the potential leaving carbonium ion and steric factors. Since dealkylation has been shown to be favored in DDC analogues where R = isopropyl, benzyl, cyclohexyl, or cyclohexenyl (21) (Table 1), it was thought that these dihydropyridines would be of interest to study from the viewpoint of decreased hepatic ferrochelatase activity and suicidal destruction of cytochrome P-450. Retention of the alkyl group during oxidation is favored in DDC analogues where R = methyl, ethyl, or propyl (21) and it was considered of interest to compare these analogues with the above (R = isopropyl, benzyl, cyclohexyl, and cyclohexenyl) as hepatic ferrochelatase-reducing agents and as agents capable of causing the suicidal destruction of cytochrome P-450.

The porphyrin-inducing activity of the dihydropyridine analogues that undergo facile oxidative 4-dealkylation, viz., 4-isopropyl (IV), 4-benzyl (V), 4-(3-cyclohexenyl) (VI), and the 4-cyclohexyl (VII), has been determined previously by a qualitative method (28). The 4-isopropyl analogue (IV) was shown to have moderate potency while the remaining analogues were weakly active. These results have been confirmed in the present study (Figs. 2 and 3) using a quantitative method. The low porphyrinogenic activity of the 4-benzyl (V), 4-(3-cyclohexenyl) (VI), and the 4-cyclohexyl (VII) analogues was attributed previously to metabolic oxidative dealkylation to the 4-desalkylpyridine, viz., 3,5-diethoxycarbonyl-2,6-dimethylpyridine (Fig. 1, route 2) followed by enzymic hydrolysis to an inactive dicarboxylic acid. If this explanation is correct, then the co-administration of BNPP, an esterase inhibitor, should prevent hydrolysis of the 4-desalkylpyridine and result in enhanced por-

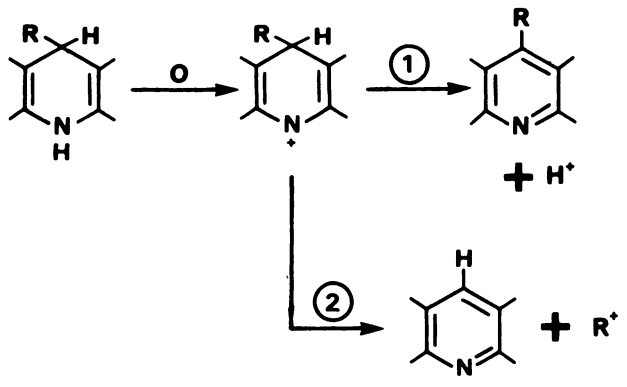


FIG. 1. *Possible routes of oxidation of 4-alkyldihydropyridines to pyridines*

1, with loss of 4-proton at position 4; 2, with loss of the 4-alkyl group as a carbonium ion.

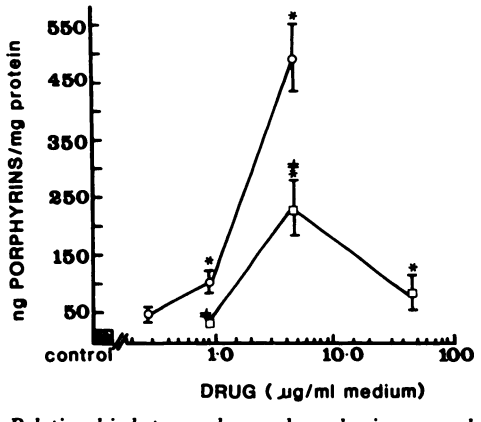


FIG. 2. *Relationship between dose and porphyrin accumulation 24 hr after the addition of DDC (O) and the 4-isopropyl analogue of DDC (□) to chick embryo liver cell culture*

Each point represents the mean ± standard deviation of five determinations. *, significant difference from control by the Newman-Keuls test ($p \leq 0.05$). ‡, significantly different from response obtained at the same dose of DDC by Newman-Keuls test ($p \leq 0.05$). The control standard deviation was ±3.1.

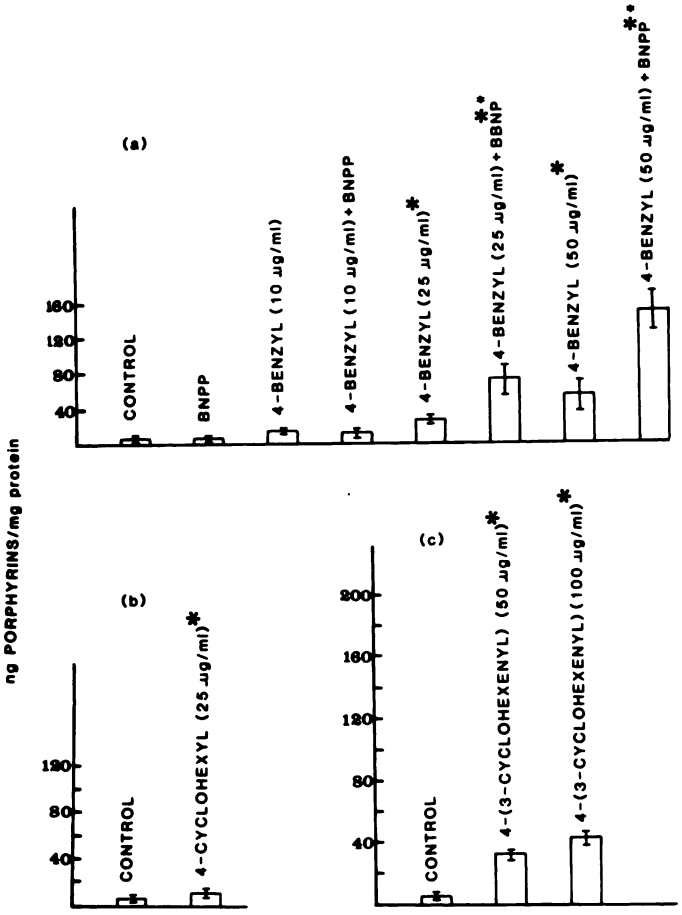


FIG. 3. *Porphyrin accumulation 24 hr after the addition of (a) the 4-benzyl analogue of DDC in the absence and presence of BNPP (10 µg/ml of medium), (b) 4-cyclohexyl, and (c) 4-(3-cyclohexenyl) analogues of DDC to chick embryo liver cell culture*

Each bar represents the mean of four determinations ± standard deviation. *, significantly different from control by Newman-Keuls test ($p \leq 0.05$). *, significantly different from the value obtained for the drug in the absence of BNPP by Newman-Keuls test ($p \leq 0.05$).

phyrinogenic activity. The 4-desalkylpyridine has previously been shown to be inactive alone but to exhibit marked activity in the presence of BNPP (20). The coadministration of BNPP with the 4-benzyl analogue of DDC resulted in enhancement of porphyrinogenic activity (Fig. 3a); no enhancement was noted with 4-cyclohexyl or 4-(3-cyclohexenyl) analogues (results not shown). The previous hypothesis to explain the low activity of these analogues appears to be tenable at least in part, in the case of the 4-benzyl analogue but not in the case of the 4-cyclohexyl or 4-(3-cyclohexenyl) analogues.

The ferrochelatase-reducing activities of DDC and its analogues were compared at doses of 0.1, 0.3, and 1.0 µg/ml of the chick embryo liver cell culture medium (Table 2). De Matteis *et al.* (3) showed that the dose of DDC required to inhibit hepatic ferrochelatase was considerably lower than that required for porphyrinogenic activity in rats and mice. Similar observations have been made in the chick embryo liver (5) and chick embryo liver cell culture (20). For this reason, the doses of DDC analogues chosen to observe ferrochelatase-reducing effects were considerably lower than those used to observe porphyrinogenic effects (Figs. 2–4). DDC and the 4-ethyl analogue produced a dose-related inhibition of ferrochelatase activity while the 4-propyl analogue showed the same ferrochelatase-inhibitory activity at all three doses. The ferrochelatase-inhibitory activity of these compounds is in accord with the finding that these DDC analogues give rise to *N*-alkylprotoporphyrin IX derivatives in rodent liver (14, 17). The inactivity of the 4-isopropyl and the 4-benzyl analogues is in accord with the finding that these two analogues do not give rise to isolable *N*-alkylprotoporphyrin IX derivatives in rat liver (17) or rat hepatocytes (18). The 4-cyclohexenyl and 4-

TABLE 2

Ferrochelatase-reducing activities of dihydropyridine analogues of DDC in chick embryo liver cell culture

Dihydropyridine analogues	Compound No.	Dose	Ferrochelatase activity[a]
		µg/ml medium	*nmol mesoheme/mg protein*
DDC	I	0	3.54 ± 0.82
		0.1	0.711 ± 0.03[b]
		1.0	0.414 ± 0.13[c]
		0	2.51 ± 0.38
		0.3	0.381 ± 0.06[d]
4-Ethyl	II	0	3.22 ± 0.20
		0.1	1.59 ± 0.33[b]
		0.3	0.874 ± 0.25[c]
		1.0	0.398 ± 0.10[c]
4-Propyl	III	0	3.25 ± 0.15
		0.1	1.89 ± 0.50[b]
		0.3	1.64 ± 0.25[b]
		1.0	1.54 ± 0.19[b]
4-Isopropyl	IV	0	3.21 ± 0.29
		0.1	3.80 ± 0.54
		0	2.51 ± 0.38
		0.3	2.41 ± 0.33
		0	2.58 ± 0.19
		0.3	2.92 ± 0.53
4-Benzyl	V	0	3.54 ± 0.82
		0.1	3.72 ± 0.58
		1.0	3.50 ± 0.41
		0	2.51 ± 0.38
		0.3	3.20 ± 0.14
4-(3-Cyclohexenyl)	VI	0	3.21 ± 0.29
		0.1	3.15 ± 0.23
		0	2.51 ± 0.38
		0.3	2.27 ± 0.54
		0	2.58 ± 0.19
		1.0	2.29 ± 0.33
4-Cyclohexyl	VII	0	3.63 ± 0.22
		0.1	3.43 ± 1.12
		0.3	3.48 ± 0.54
		1.0	2.64 ± 0.43

[a] Values given are means (± standard deviation) for four determinations.

[b] Significantly different from control, $p \leq 0.05$; one-way analysis of variance and difference between groups were tested with Newman-Keuls test.

[c] Significantly different from preceding group, $p \leq 0.05$; one-way analysis of variance and differences between groups were tested by the Newman-Keuls test.

[d] Significantly different from control, $p \leq 0.05$, Student's *t*-test.

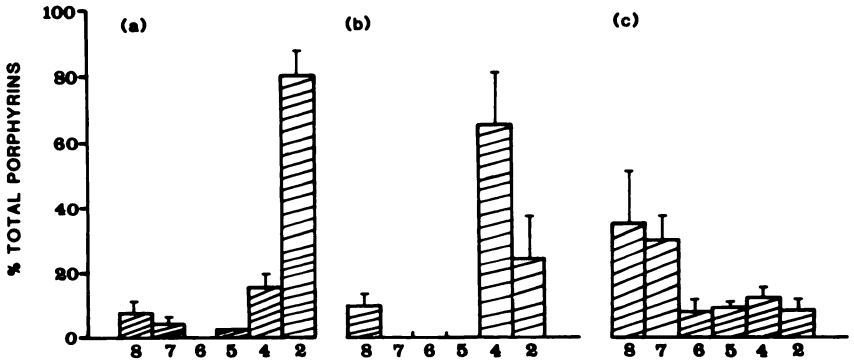


FIG. 4. *Porphyrin patterns obtained 24 hr after the addition of (a) DDC (I) (5 μg/ml of medium), (b) 4-isopropyl analogue (V) (10 μg/ml of medium), and (c) 4-benzyl analogue (VI) (25 μg/ml of medium) in the presence of BNPP (10 μg/ml) to chick embryo liver cell cultures*

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 deviation) of five determinations in one experiment and the results were confirmed in three additional experiments. Absolute values of total porphyrins shown here were as follows: (a) 1230.7, (b) 183.4, and (c) 926 pmol/mg of protein. Control levels were 87.8 ± 35.5 pmol/mg of protein; uroporphyrin was the main porphyrin observed in controls (60%) acompanied by coproporphyrin (40%).

TABLE 3

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

Dihydropyridine analogues	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*
DDC	I	0	0.268 ± 0.006	0.635 ± 0.028		
		125	0.210 ± 0.010[b]	0.567 ± 0.037[b]	0.058	0.068
4-Ethyl	II	0	0.262 ± 0.026	0.678 ± 0.031		
		125	0.148 ± 0.024[b]	0.489 ± 0.039[b]	0.114	0.189
4-Propyl	III	0	0.268 ± 0.006	0.635 ± 0.028		
		125	0.169 ± 0.008[b]	0.490 ± 0.012[b]	0.099	0.145
4-Isopropyl	IV	0	0.249 ± 0.031	0.621 ± 0.021		
		120	0.161 ± 0.010[b]	0.485 ± 0.010[b]	0.085	0.136
4-Benzyl	V	0	0.268 ± 0.006	0.635 ± 0.028		
		125	0.206 ± 0.027[b]	0.584 ± 0.025[b]	0.062	0.051
4-(3-Cyclohexenyl)	VI	0	0.232 ± 0.022	0.510 ± 0.020		
		125	0.143 ± 0.012[b]	0.419 ± 0.015[b]	0.089	0.091
4-Cyclohexyl	VII	0	0.232 ± 0.022	0.510 ± 0.020		
		125	0.147 ± 0.019[b]	0.428 ± 0.011[b]	0.085	0.082
4-Phenyl	VIII	0	0.234 ± 0.029	0.623 ± 0.022		
		125	0.227 ± 0.011	0.608 ± 0.014		
Desmethyl-DDC	IX	0	0.234 ± 0.029	0.623 ± 0.022		
		125	0.233 ± 0.015	0.594 ± 0.009		
OX-DDC		0	0.234 ± 0.029	0.623 ± 0.022		
		125	0.236 ± 0.011	0.608 ± 0.029		

[a] Values given are means (± standard deviation) for four determinations.

[b] Significantly different from control, $p \leq 0.05$, Student's *t* test.

cyclohexyl analogues are devoid of ferrochelatase-inhibitory activity, presumably because they do not give rise to *N*-alkylprotoporphyrin IX derivatives in the liver. Further studies are required to determine if this is the case.

DDC causes the accumulation of protoporphyrin as the major porphyrin accompanied by a small amount of coproporphyrin (20). This finding has been confirmed in the present study (Fig. 4a). The porphyrin pattern produced by DDC is consistent with the ability of DDC to inhibit ferrochelatase. The porphyrin pattern produced by the 4-isopropyl analogue (IV) is consistent with its inability to inhibit ferrochelatase. Thus, coproporphyrin rather than protoporphyrin is the major porphyrin (Fig. 4b). The porphyrin pattern produced by the 4-benzyl analogue (V) in the presence of BNPP (Fig. 4c) is consistent with its inability to inhibit ferrochelatase. Thus, uroporphyrin and heptacarboxylic porphyrin rather than protoporphyrin were the main porphyrins. It was concluded from these studies that branching of the carbon chain of the 4-alkyl group at the carbon directly attached to the dihydropyridine ring was incompatible with ferrochelatase-inhibitory activity. Moreover, a 4-phenyl (20) or 4-benzyl substituent (Table 2) is also incompatible with ferrochelatase-inhibitory activity. These conclusions are compatible with the suggestion of Ortiz de Montellano and Correia (19) that a primary carbon substituent without conjugating groups is required at posi-

tion 4 of the dihydropyridine ring in order for heme alkylation to occur.

The inability of the 4-isopropyl (IV), 4-benzyl (V), 4-(3-cyclohexenyl) (VI), and 4-cyclohexyl (VII) analogues to inhibit ferrochelatase explains, at least in part, why their porphyrinogenic activity is markedly less than that of DDC. According to current ideas, DDC owes its porphyrinogenic action to: (a) ferrochelatase inhibition via *N*-MePP formation; (b) a second mechanism, whereby an elevation of ALAS (EC 2.3.1.37) is induced, independent of inhibition of heme biosynthesis or of 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 ALAS and 2) increased synthesis of the mRNA for apocytochrome P-450, leading to increased synthesis of apocytochrome P-450 with increased utilization of heme from a "regulatory heme pool" for the synthesis of cytochrome P-450 (29). It has been pointed out that all drugs that induce ALAS by this second mechanism have the property of lipid solubility (29). The DDC analogues of lower potency presumably owe their porphyrinogenic activity solely to ALAS induction by the second mechanism.

Our next studies were directed to determining the *in vitro* effects of DDC analogues on hepatic cytochrome P-450. Desmethyl-DDC (IX) lacks a 4-alkyl substituent and therefore cannot form an *N*-alkylprotoporphyrin IX. Thus, its inability to inhibit ferrochelatase (20) is understandable. The present demonstration (Table 3) that it does not cause destruction of cytochrome P-450 accords with its inability to transfer a 4-alkyl substituent and to form a *N*-alkylprotoporphyrin IX capable of inhibiting ferrochelatase. OX-DDC, lacking a dihydropyridine structure, does not have a labile 4-alkyl substituent. Thus, its inability to inhibit ferrochelatase (20) is readily understandable and it was anticipated that it would not cause destruction of cytochrome P-450; this was found to be the case (Table 3). The 4-phenyl analogue (IX) does not inhibit ferrochelatase (20) and it was anticipated that it would not cause destruction of cytochrome P-450; this was found to be the case (Table 3).

The next group investigated, viz., DDC, 4-ethyl (II), and 4-propyl (III), were potent ferrochelatase inhibitors, a fact which accorded with their demonstrated ability to give rise to *N*-alkyl protoporphyrin IX derivatives in rodent liver (14, 17, 18). It was therefore anticipated that these three compounds would cause hepatic cytochrome P-450 destruction; this was found to be the case (Table 3). Closely similar results were obtained by Augusto *et al.* (17) with the 4-ethyl and 4-propyl analogues using hepatic microsomes from phenobarbital-pretreated male rats rather than hepatic microsomes from chick embryo liver employed in the present study. A difference was observed, however, with the cytochrome P-450-destructive effect of DDC in the two studies. While Augusto *et al.* (17) observed barely discernible cytochrome P-450 destruction, in the present study, DDC induced significant destruction of cytochrome P-450. A possible explanation for the difference in results may reside in the differences in the isozymes of cytochrome P-450 in the phenobarbital-induced rat liver and in uninduced chick embryo hepatic microsomes. This explanation is supported by a recent report of Brooker *et al.* (30) that no synthesis of the phenobarbital-inducible form of cytochrome P-450 could be detected in uninduced chick embryo hepatic microsomes.

The third group of DDC analogues studied were those that undergo facile oxidative 4-dealkylation, viz., 4-isopropyl (IV), 4-benzyl (V), 4-(3-cyclohexenyl) (VI), and 4-cyclohexyl (VII). These analogues were devoid of ferrochelatase-inhibitory activity and it was anticipated that they would have no cytochrome P-450-destructive activity. However, contrary to expectation, these analogues were found to have marked cytochrome P-450-destructive activity (Table 3). Closely similar results were obtained with the 4-isopropyl (IV) and 4-benzyl (V) analogues by Augusto *et al.* (17) using hepatic microsomes from phenobarbital-treated Sprague-Dawley male rats and by De Matteis *et al.* (18) using rat hepatocytes and mouse hepatic microsomes.

Augusto *et al.* (17) suggested that these two analogues (IV and V) caused protein rather than heme alkylation. If protein rather than heme alkylation occurs with analogues IV, V, VI, and VII, an explanation would be provided for cytochrome P-450 destruction in the absence of ferrochelatase inhibition. To determine whether this explanation was valid, heme levels were measured after incubation of the DDC analogues with chick embryo hepatic microsomes. The results (Table 3) show that destruction of cytochrome P-450 by DDC analogues I–VII was paralleled by heme loss; analogues VIII, IX, and OX-DDC, which did not cause cytochrome P-450 destruction, did not cause heme loss. Thus, despite the lack of ferrochelatase-inhibitory activity of the 4-isopropyl (IV), 4-benzyl (V), 4-(3-cyclohexenyl) (VI), and 4-cyclohexyl (VII) analogues, these compounds cause the degradation of the heme moiety of cytochrome P-450, and it is not necessary to invoke alkylation of the protein moiety of cytochrome P-450 as an explanation of their destructive activity. In agreement with these findings, De Matteis *et al.* (18) have reported that the cytochrome P-450-destructive action of the 4-isopropyl (IV) analogue is accompanied by heme loss in mouse liver microsomes. Since heme destruction is not accompanied by ferrochelatase-inhibitory effects with analogues IV–VII and since *N*-alkylporphyrins have not been isolated following administration of the 4-isopropyl (IV) and 4-benzyl (V) analogues (17, 18), it is unlikely that heme destruction with analogues IV–VII leads to *N*-alkylporphyrin formation.

In summary, we have shown that in one group of DDC analogues an inability to decrease hepatic ferrochelatase activity corresponded to an inability to cause cytochrome P-450 and heme destruction. In a second group of DDC analogues, the ability to decrease hepatic ferrochelatase activity corresponded to the ability to cause cytochrome P-450 and heme destruction. These results support the idea that the protoporphyrin IX moiety of *N*-alkylprotoporphyrin IX originates from the heme moiety of cytochrome P-450. A third group of DDC analogues causes cytochrome P-450 destruction despite an inability to reduce ferrochelatase activity. In this third group, the

heme moiety of cytochrome P-450 is probably degraded to products other than *N*-alkylporphyrins. It will therefore be of considerable interest to determine the degradative pathway followed by the heme moiety of cytochrome P-450 following destruction by this group of DDC analogues.

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