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EFFECTS OF 3-(2-PHENYLETHYL)-4-METHYLSYDNONE AND RELATED SYDNONES ON HEME BIOSYNTHESIS

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Abstract—3-[2-(2,4,6-Trimethylphenyl)thioethyl]-4-methylsydnone (TTMS) and 3-(2-phenylethyl)-4-methylsydnone (PEMS) cause mechanism-based inactivation of rat hepatic microsomal cytochrome P-450 and the formation of *N*-alkylprotoporphyrins in rat liver. In the present study, we have shown that both TTMS and PEMS cause mechanism-based inactivation of chick embryo hepatic microsomal cytochrome P-450. TTMS also caused the inhibition of ferrochelatase activity, the accumulation of protoporphyrin IX, and an increase in the activity of δ-aminolevulinic acid synthase in chick embryo liver cell culture. PEMS was devoid of effect on ferrochelatase activity, porphyrin accumulation, and δ-aminolevulinic acid synthase activity. There are two possible explanations for the lack of effect of PEMS on heme biosynthesis: (1) the ring-A- and/or ring-B-substituted regioisomers of the *N*-phenylethyl- and *N*-phenylethenylprotoporphyrins which are produced during the mechanism-based inactivation of cytochrome P-450 by PEMS are too bulky to fit into the active site of ferrochelatase to inhibit its activity, in contrast to the *N*-vinylprotoporphyrin formed from TTMS; and (2) the *N*-alkylprotoporphyrins produced consist of the ring-C- and/or ring-D-substituted regioisomers, which are not inhibitors of ferrochelatase activity.

Hepatic heme biosynthesis is regulated by the first enzyme involved in heme biosynthesis, δ-aminolevulinic acid synthase (ALAS§) (EC 2.3.1.37) [1]. The activity of ALAS is controlled via feedback repression by the end-product heme, through a "regulatory free heme pool" [1]. Heme is currently believed to mediate this control: (1) by inhibiting transcription of the ALAS gene [2]; (2) by inhibiting translation of the ALAS mRNA [3, 4]; and/or (3) by slowing translocation of the inactive pre-ALAS from the cytosol into the mitochondrion, where mature ALAS is active [5, 6]. Heme levels in hepatocytes can be lowered in several ways. Two ways relevant to this study are: (1) by inhibition of one of the heme biosynthetic enzymes, for example ferrochelatase (FC) and (2) by destroying the heme of cytochrome P-450. This leaves a free apocytochrome P-450, which can combine with a new heme moiety and thereby drain the free heme pool.

Compounds such as 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC) (Fig. 1a) interfere with heme biosynthesis. 4-Ethyl DDC causes mechanism-based destruction of cytochrome P-450 [7] and during metabolism by cytochrome P-450 an ethyl radical is released from 4-ethyl DDC [8]. This radical is believed to bind to one of the four nitrogens of cytochrome P-450, producing *N*-ethylprotoporphyrin IX (N-EtPP) (Fig. 2) [8]. N-EtPP is a potent inhibitor of FC activity [9–11] and, therefore, protoporphyrin IX accumulates [11, 12]; ALAS activity is also increased [13].

Studies on the potential anti-arthritic agent 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS) (Fig. 1b) were halted upon the discovery that TTMS produces hepatic protoporphyrin IX accumulation [14], suggesting that TTMS may act in a manner similar to 4-ethyl DDC [15]. TTMS causes mechanism-based destruction of cytochrome P-450 in rat liver [16]; it has been postulated that during this metabolism a 2-carbon fragment of TTMS is transferred to one of the nitrogens of the heme moiety of cytochrome P-450, as illustrated (Fig. 3), producing *N*-vinylprotoporphyrin IX (N-ViPP) (Fig. 2) [16]. N-ViPP formation is believed to be responsible for the inhibition of FC activity observed when TTMS is added to chick embryo liver cell culture [17]; accumulation of protoporphyrin IX is also observed in the culture system. An analogue of TTMS, 3-benzyl-4-phenylsydnone (BPS) (Fig. 1c) does not inhibit FC activity but does cause porphyrin accumulation in chick embryo liver cell culture [17]. The porphyrins which accumulate following the administration of BPS are uroporphyrin and hepta- and hexacarboxylic acid porphyrins.

A third analogue of TTMS, 3-(2-phenylethyl)-4-methylsydnone (PEMS) (Fig. 1d), has been shown to cause mechanism-based inactivation of rat hepatic microsomal cytochrome P-450 and the formation of *N*-phenylethyl- and *N*-phenylethenylprotoporphyrins in rat liver [18]. With these parallels to TTMS and 4-ethyl DDC, our objectives were to answer the following questions: (a) Does PEMS decrease FC activity in chick embryo liver cell culture? (b) Does

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§ Abbreviations: ALAS, δ-aminolevulinic acid synthase; FC, ferrochelatase; 4-ethyl DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; TTMS, 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone; BPS, 3-benzyl-4-phenylsydnone; PEMS, 3-(2-phenylethyl)-4-methylsydnone; and N-EtPP, *N*-ethylprotoporphyrin IX.

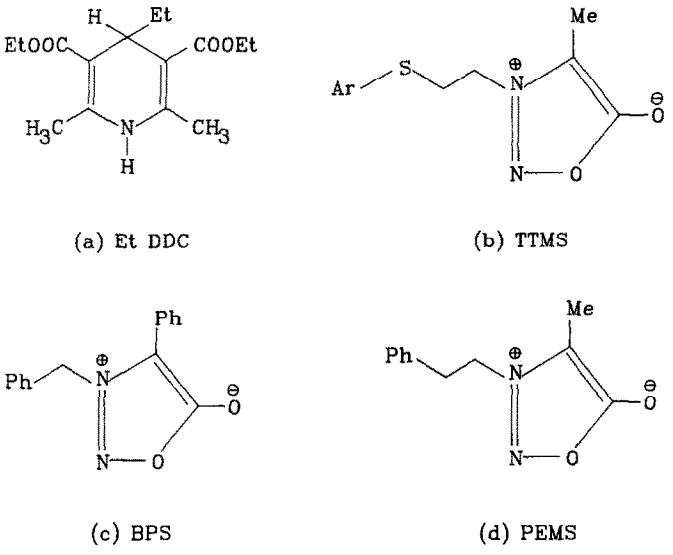

Fig. 1. Structures of the compounds used in this study: (a) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC or Et DDC); (b) 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS) (Ar = 2,4,6-trimethylphenyl); (c) 3-benzyl-4-phenylsydnone (BPS); and (d) 3-(2-phenylethyl)-4-methylsydnone (PEMS).

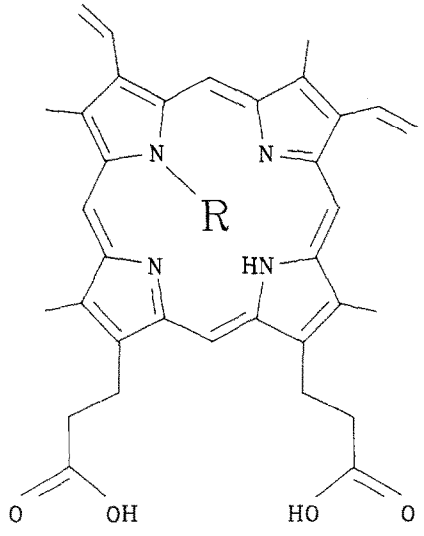

Fig. 2. Structures of the *N*-alkylprotoporphyrins: N-EtPP, R = CH_2CH_3; N-ViPP, R = $CH{=}CH_2$; N-phenylethylPP, R = $CH_2CH_2(C_6H_6)$; and N-phenylethenylPP, R = $CH{=}CH(C_6H_6)$.

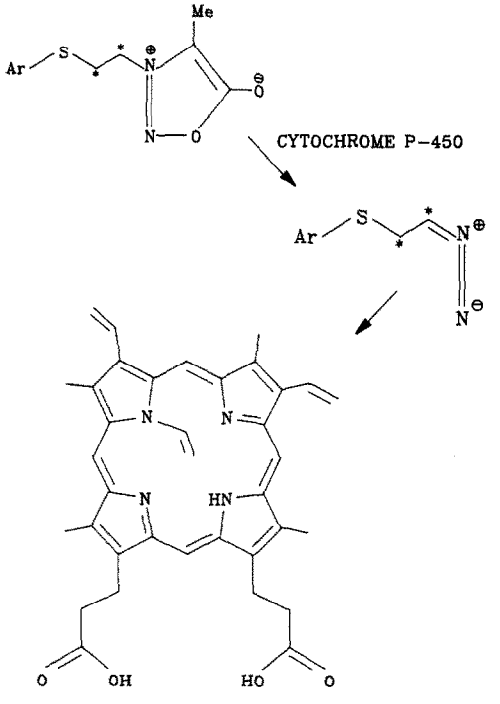

Fig. 3. Proposed mechanism of formation of N-ViPP from TTMS during the metabolism of TTMS by cytochrome P-450. (Ar = 2,4,6-trimethylphenyl.)

PEMS increase protoporphyrin IX or any other porphyrin in chick embryo liver cell culture? (c) How do the effects of PEMS on FC activity and pattern of porphyrin accumulation compare to those of TTMS and BPS? (d) Does TTMS, BPS, or PEMS affect ALAS activity in chick embryo liver cell culture? (e) Does TTMS, BPS or PEMS cause destruction of cytochrome P-450 in chick embryo hepatic microsomes? 4-Ethyl DDC was included in all studies as a positive control. A series of concentrations of each sydnone were tested.

MATERIALS AND METHODS

Materials. [2,3-^{14}C]Succinic acid (40–80 μCi/mmol) was obtained from New England Nuclear (Mississauga, Ontario). Mesoporphyrin IX, coproporphyrin I, hepta-, hexa-, and pentacarboxylic acid

porphyrins and uroporphyrin I were obtained from Porphyrin Products (Logan, UT). 4-Ethyl DDC was synthesized in the laboratory as previously described [19, 20]. TTMS, BPS, and PEMS also were synthesized as previously described [16, 18].

Cell culture. The cell culture technique has been described previously [21, 22]. Briefly, the livers were removed from 18-day-old White Leghorn chicken embryos. Hepatocytes were cultured in Waymouth 705/1A medium, supplemented with thyroxine and insulin and maintained in plastic culture dishes. Specific conditions for individual assays will be outlined below.

Ferrochelatase activity. Hepatocytes were cultured in 10-cm dishes, containing 15 mL of medium. Medium was changed 24 and 42 hr after seeding. At this point drugs were added in 95% EtOH, along with an EtOH control (maximum volume added was 10 μL). Cells were harvested 6 hr after drug administration, and ferrochelatase activity was determined using a cellular homogenate as the source of ferrochelatase, with mesoporphyrin IX and ferrous iron as substrates [22, 23]. The production of mesoheme was quantitated using the pyridine mesohemochromogen assay [24]; the results are expressed as nanomoles mesoheme formed per 10 minutes per milligram of protein.

Pattern of porphyrin accumulation. Hepatocytes were cultured in 6-cm dishes containing 5 mL of medium. Medium was changed 24 hr after seeding and drugs were added in 95% EtOH, along with an EtOH control (maximum volume added was 10 μL). Total porphyrins were measured 24 hr after drug administration [24] using an HPLC system with a fluorescence detector [25, 26]. Results are expressed as picomoles of each porphyrin per milligram of protein.

δ-Aminolevulinic acid synthase activity. Hepatocytes were cultured in 10-cm dishes. Medium was changed 24 and 44 hr after seeding. Drugs were added in 95% EtOH, along with an EtOH control (maximum volume added 10 μL), and cells were harvested at 0, 3, 6, 12 and 24 hr after drug addition. The activity of δ-aminolevulinic acid synthase was measured by incubating a cellular homogenate as the source of δ-aminolevulinic acid synthase, with [2,3-^{14}C]succinic acid, pyridoxal phosphate, glycine and ATP [26, 27]. The labeled ALA which was produced was isolated by ion-exchange chromotography and converted to a pyrrole with acetylacetone; the pyrrole was quantitated by liquid scintillation counting. Results are expressed as picomoles ALA per milligram of protein per 30 minutes.

Cytochrome P-450 levels. The method of measuring cytochrome P-450 was adapted from that used by Omura and Sato [28] for rat liver microsomes and has been described previously [19, 20]. Briefly, microsomes were prepared from livers of 18-day-old chick embryos by differential centrifugation and stored at −70° until used (not longer than 2 weeks). The microsomes were resuspended in 0.1 mM potassium phosphate buffer, pH 7.4, containing 1.5 mM EDTA, to approximately 1 mg/mL microsomal protein. Microsomes were incubated in the presence of a 1 mM concentration 4-ethyl DDC, TTMS, BPS, or PEMS (in 20 μL EtOH) with or without NADPH (2 mM) for 30 min at 37° in a shaking water bath. An EtOH plus NADPH control was run concurrently. The reaction was stopped by placing the samples on ice. Sodium dithionite (several milligrams) was added, and the reaction mixture was divided between sample and reference cuvettes of a Unicam SP8-100 spectrometer and the CO-binding spectrum of cytochrome P-450 was determined [28].

Protein. The amount of protein was measured using the method of Lowry *et al.* [29]. Bovine serum albumin was used as the standard.

Statistics. A randomized one-way analysis of variance test was used to detect significant differences in FC activity and porphyrin pattern. If a significant F ratio at the 0.05 level was obtained, a Newman–Keuls test was used to detect the means which differed significantly from each other. A two-way ANOVA was used to indicate the presence of significant differences in ALAS activity, and randomized ANOVA tests were used to locate the significant differences. A repeated measures ANOVA test was used to detect significant differences in cytochrome P-450 levels, with a Neuman–Keuls test to detect the significantly different means.

RESULTS AND DISCUSSION

TTMS has been shown to cause a mechanism-based inactivation of cytochrome P-450 and the accumulation of N-ViPP in rat liver [16], and to produce inhibition of ferrochelatase activity and accumulation of protoporphyrin IX and coproporphyrin in chick embryo hepatocyte cultures [17]. It is believed that, during the metabolism of TTMS by cytochrome P-450, a radical is released from TTMS which binds to the heme of cytochrome P-450 producing N-ViPP [16]. N-ViPP is thought to be a potent inhibitor of FC activity; heme levels could be lowered by this inhibition of FC and destruction of cytochrome P-450 heme. This decrease in heme levels would then result in derepression of ALAS activity. PEMS has been shown to cause a mechanism-based inactivation of cytochrome P-450 and the formation of *N*-phenylethyl- and *N*-phenylethenylprotoporphyrin IX in rat liver [18]. The purposes of this paper were: to determine whether PEMS would affect heme biosynthesis (FC activity, ALAS activity and porphyrin accumulation); to compare these effects to those caused by two other sydnones, TTMS and BPS; to examine the effects of higher concentrations of TTMS and BPS on FC activity and the pattern of porphyrin accumulation; and to examine the effects of TTMS, BPS, and PEMS on chick embryo hepatic microsomal cytochrome P-450.

Effect of sydnones on the activity of ferrochelatase in chick embryo hepatocyte culture. To determine the effect on ferrochelatase, concentrations of 0.004, 0.04, 0.4, and 4 μM TTMS or 0.04, 0.4, 4, and 40 μM PEMS or 4 μM 4-ethyl DDC were added to hepatocytes in culture and the activity of FC was determined. 4-Ethyl DDC, a potent porphyrinogenic agent serving as a positive control throughout these studies, lowered FC activity almost completely (Fig. 4). This lowering of FC activity was attributed to the

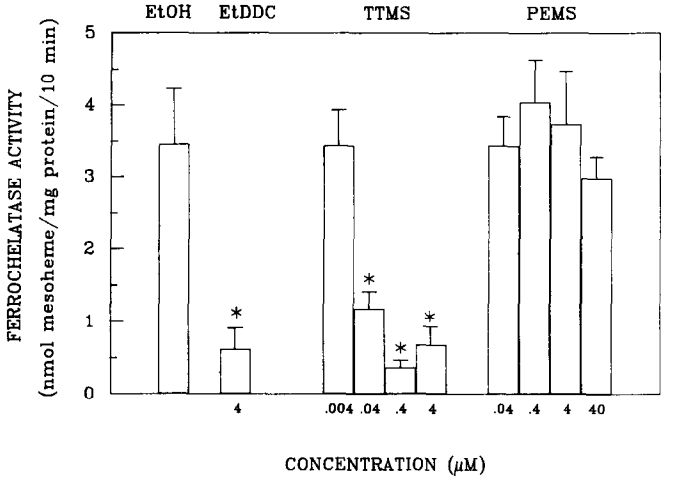

Fig. 4. Activity of ferrochelatase in chick embryo liver cell culture at 6 hr after administration of 4-ethyl DDC (EtDDC), TTMS, and PEMS. 4-Ethyl DDC, TTMS, and PEMS were given at the concentrations indicated. Each value is the mean (±SD) of four determinations. Results were confirmed in additional experiments. An asterisk (*) indicates a decrease from control levels at $P \leq 0.05$. Control levels were 3.46 nmol mesoheme per 10 min per mg protein.

formation of N-EtPP during the metabolism of 4-ethyl DDC by cytochrome P-450. TTMS, as previously seen [17], lowered FC activity (Fig. 4) to levels similar to those observed with 4-ethyl DDC, indicating a similar efficacy of the two compounds. In a previous study, using lower doses of TTMS, the activity of FC was maximally lowered to only 42% of control activity as opposed to 10% of control, as observed with the higher doses used in this study. The inhibition of FC activity was attributed to the N-ViPP which is formed during the metabolism of TTMS by cytochrome P-450. BPS did not alter the activity of FC in this study (results not shown), as was expected from an earlier study [17], even at the highest dose tested. BPS lacks a thioethyl fragment in the 3-position and, therefore, cannot be activated to form a FC-inhibitory N-ViPP in the way that TTMS can be [17]. PEMS did not affect FC activity (Fig. 4), even up to doses of 40 μM, in spite of the fact that the formation of *N*-alkylporphyrins was observed in rat livers.

Effect of sydnones on the pattern of porphyrin accumulation in chick embryo hepatocyte culture. A series of concentrations of the sydnones (0.04, 0.4, 4 and 40 μM), or a concentration of 12 μM 4-ethyl DDC, were incubated with hepatocytes for 24 hr, and the patterns of porphyrin accumulation were determined. 4-Ethyl DDC produced predominantly protoporphyrin, with some coproporphyrin (Fig. 5); this was due to the inhibition of FC by N-EtPP. TTMS produced a similar pattern, with a significant increase in protoporphyrin accumulation at concentrations of 4 and 40 μM TTMS (Fig. 5), along with an increase in coproporphyrin levels at 40 μM TTMS. The amount of porphyrins accumulating was similar to that seen with 4-ethyl DDC, indicating a similar efficacy of TTMS and 4-ethyl DDC in promoting excess porphyrin accumulation. The accumulation of protoporphyrin could be attributed to the inhibition of FC caused by N-ViPP. BPS produced a strikingly different pattern: uroporphyrin, hepta- and hexacarboxylic acid porphyrins were the only porphyrins to accumulate to a significant level (Fig. 5); this occurred at the highest concentration of BPS only. There was no significant accumulation of any porphyrins with PEMS, even at the highest dose (Fig. 5). From Fig. 5, it appears that there was an accumulation of porphyrins; however, this increase was not significantly higher than control levels of porphyrins.

Effect of sydnones on the activity of δ-aminolevulinic acid synthase in chick embryo hepatocyte culture. Concentrations of 4 μM of the sydnones or 4-ethyl DDC were compared for their effects on the rate-limiting enzyme of heme biosynthesis, ALAS. A concentration of 4 μM was chosen because this was the lowest concentration that produced both maximal inhibition of FC activity and maximal accumulation of porphyrins. If the compounds are lowering the levels of free heme in hepatocytes, then an increase in the activity of ALAS would be expected, as the repression of ALAS by heme would be removed. 4-Ethyl DDC produced an increase in ALAS activity over time, with a peak activity of 817% of control levels occurring 12 hr after administration; activity returned to control levels by 24 hr after treatment (Fig. 6). This confirms earlier results [13]. It also reflects the accumulation of protoporphyrin IX which was seen (Fig. 5). TTMS produced changes in ALAS activity similar to those seen with 4-ethyl DDC (Fig. 6). A peak activity of 551% of control levels was reached at 12 hr after administration of TTMS; activity returned to control by 24 hr after treatment. TTMS is believed to lower heme levels by inhibiting the activity of FC, as seen above (Fig. 4). Despite the fact that BPS did not lower FC activity, BPS produced similar changes in ALAS activity over time. The peak activity of 392% of control levels obtained at 12 hr after administration of BPS was not significantly different from

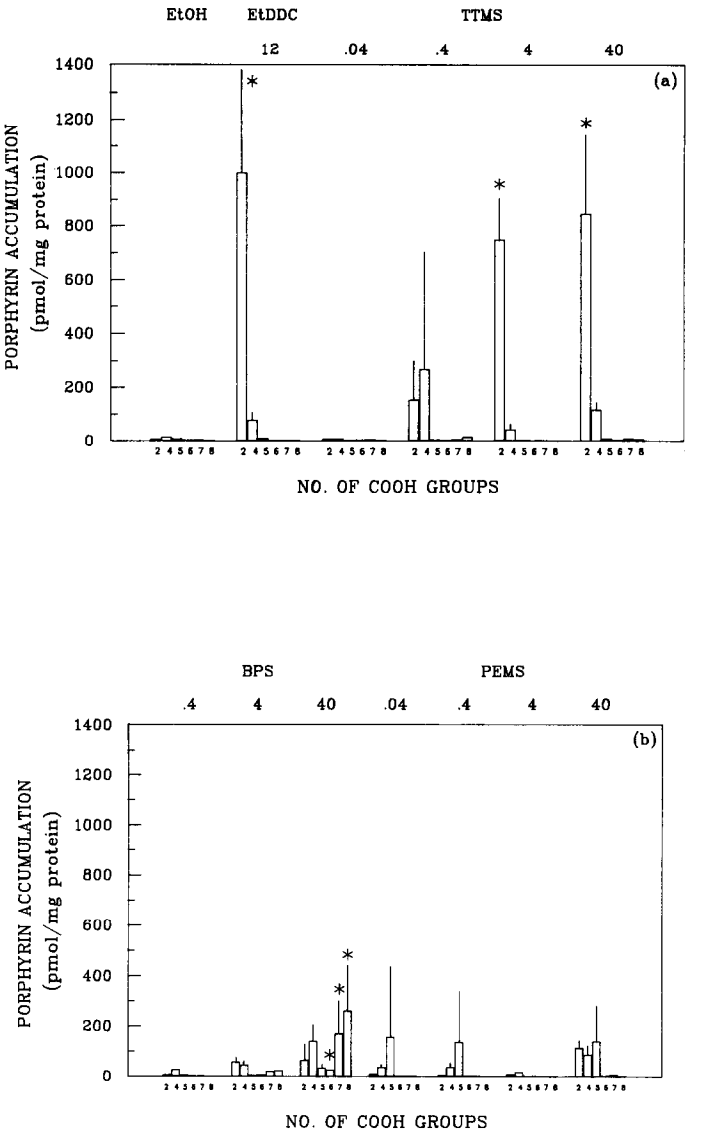

Fig. 5. Pattern of porphyrin accumulation in chick embryo liver cell culture 24 hr after the administration of 4-ethyl DDC (EtDDC), TTMS, BPS and PEMS at the concentrations indicated above the clusters of bars. Each value is the mean (±SD) of four determinations. Results were confirmed in additional experiments. An asterisk (*) indicates an increase over control levels at $P \leq 0.05$. Control levels were: 7.1 pmol protoporphyrin IX/mg protein, 16.4 pmol coproporphyrin/mg protein, 13.5 pmol pentacarboxylic acid porphyrin/mg protein, 4.0 pmol hexacarboxylic acid porphyrin/mg protein, 4.4 pmol heptacarboxylic acid porphyrin/mg protein, and 2.5 pmol uroporphyrin/mg protein. Key: 2 = protoporphyrin IX, 4 = coproporphyrin, 5 = pentacarboxylic acid porphyrin, 6 = hexacarboxylic acid porphyrin, 7 = heptacarboxylic acid porphyrin, and 8 = uroporphyrin.

the peak seen with TTMS; activity returned to control levels by 24 hr as with the other compounds (Fig. 6). PEMS did not alter significantly the activity of ALAS at any time during the 24-hr period studied (Fig. 6), a fact which accords with the lack of porphyrin accumulation (Fig. 5).

Effect of sydnones on cytochrome P-450 levels in chick embryo hepatic microsomes. Chick embryo hepatic microsomes were incubated with a concentration of 1 mM 4-ethyl DDC, TTMS, BPS, or PEMS for 30 min and the levels of cytochrome P-450 were determined. 4-Ethyl DDC produced a 25% decrease in cytochrome P-450 levels (Table 1), similar to previously published results [19]. This decrease in cytochrome P-450 levels was a result of the destruction of the heme of cytochrome P-450 through the formation of N-EtPP; N-EtPP has been isolated from livers of chick embryos treated with 4-ethyl DDC [30]. TTMS produced a similar decrease (26%) in the levels of cytochrome P-450 (Table 1). This

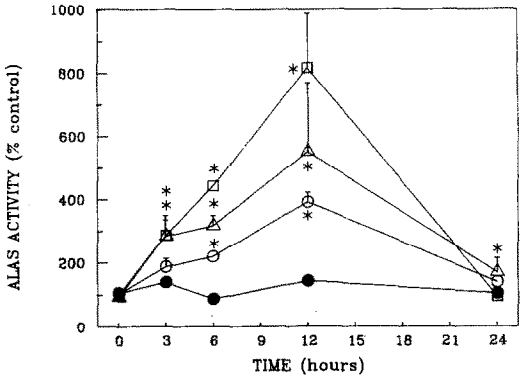

Fig. 6. Activity of δ-aminolevulinic acid synthase expressed as the percent of a 95% ethanol control at 0, 3, 6, 12 and 24 hr after administration of 4-ethyl DDC (□), TTMS (△), BPS (○), and PEMS (●). The sydnones and 4-ethyl DDC were given at a concentration of 4 μM. Each point is the mean (±SD) of three determinations. Results were confirmed in additional experiments. An asterisk (*) indicates an increase over control levels at $P \leq 0.05$. Control levels of δ-aminolevulinic acid synthase activity were 102.7 nmol ALA per mg protein per 30 min.

Table 1. *In vitro* levels of chick embryo hepatic microsomal cytochrome P-450 after a 30-min incubation with a 1 mM concentration of 4-ethyl DDC, TTMS, BPS, or PEMS

Compound	NADPH (2 mM)	Cytochrome P-450 (nmol/mg protein)	% Decrease*
EtOH	+	0.280 ± 0.030	
4-Ethyl DDC	−	0.308 ± 0.052	
	+	0.211 ± 0.006†	25 ± 2
TTMS	−	0.265 ± 0.027	
	+	0.206 ± 0.022†	26 ± 8
BPS	−	0.310 ± 0.051	
	+	0.299 ± 0.037	
PEMS	−	0.309 ± 0.007	
	+	0.167 ± 0.019†	40 ± 7

Values are means ± SD for three determinations.
* Values are expressed as percent of EtOH + NADPH control.
† Significantly different from EtOH control at $P \leq 0.05$.

destruction could be attributed to the formation of N-ViPP, which has been isolated from livers of rodents treated with TTMS [16]. The presence of N-ViPP was suggested by the lowering of FC activity seen in this (Fig. 4) and a previous study [17]. BPS did not alter cytochrome P-450 levels (Table 1); this could be due to the lack of a leaving group on the BPS structure, which prevents the activation of BPS to a heme alkylating radical. PEMS produced a 40% decrease in cytochrome P-450 levels (Table 1); this destruction was not significantly different from that seen with either 4-ethyl DDC or TTMS. The destruction of cytochrome P-450 suggests the formation of an N-alkylPP, in analogy to the formation of N-EtPP with 4-ethyl DDC. The formation of both *N*-phenylethyl- and *N*-phenylethenylPP has been detected in rat liver. However, these N-alkylPPs did not appear to be able to inhibit the activity of ferrochelatase (Fig. 4).

PEMS did not affect heme biosynthesis in the chick embryo liver cell culture system, despite the formation of *N*-phenylethyl- and *N*-phenylethenylprotoporphyrin IX observed in rat livers [18]. It was anticipated that PEMS would lower the regulatory free heme pool, if not by inhibition of FC, then at least by the destruction of the heme moiety of cytochrome P-450, and lead to an increase in the activity of ALAS. There are several possible explanations for the lack of effect of PEMS on heme biosynthesis. First, the heme of cytochrome P-450 is destroyed by interaction with PEMS, but the cytochrome P-450 cannot be reconstituted by another heme moiety. This would prevent a drain on the free heme pool. Precedent for this phenomenon exists with the compound 1-aminobenzotriazole, which produces up to an 80% decrease in cytochrome P-450 levels in rats and a decrease of 50% in microsomal heme levels [31], but causes only a very slight increase in the activity of ALAS. Second, the N-alkylPPs are formed but they are not inhibitory to FC. It has been shown that the inhibitory activity of an N-alkylPP depends on both the size and the

location of the alkyl group [32]. If the group is bulky, there is less inhibition of FC; both the phenylethyl and the phenylethenyl groups are bulky. If the group is located on the C- or D-ring nitrogen of the protoporphyrin IX macrocycle, as opposed to the A- or B-ring nitrogen, there is virtually no inhibition of FC. Nuclear magentic resonance spectra of the N-phenylethyl- and N-phenylethenylPPs formed in rodent liver suggest that the major isomers of the N-alkylPPs which were formed are N-substituted on the C- and/or D-rings of the protoporphyrin IX molecule [18]. Thus, the bulkiness of the N-alkyl substituents and their predominant location on the C- and/or D-rings provide an explanation for the lack of effect of PEMS on FC.

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