product (94%, mp 221–223 °C) from i-PrOH/Et$_2$O: ^{1}H NMR (D$_2$O) δ 3.25 (m, CH$_2$CH$_2$), 4.18 (s, CH$_2$Ph), 5.25 (s, CH$_2$N), 7.20 (m, 2 Ph), 8.80 (s, Im 2 H). Anal. (C$_{19}$H$_{23}$Cl$_2$N$_3$) C, H, N, Cl.

4(5)-(1-Cyano-2-phenylethyl)imidazole (24). NaOH (5 N, 10 mL) was added to a solution of 4(5)-(cyanomethyl)imidazole[19] (10 g, 0.093 mol) and benzaldehyde (15 g, 0.14 mol) in MeOH (100 mL). After standing at room temperature for 24 h, the reaction mixture was poured into an equal volume of water to give **23** (14 g, 77%), mp 189–190 °C. Recrystallization from EtOH gave a sample: mp 190–191 °C; IR ν_{CN} 2222 cm^{-1}; UV λ_{max} 324 nm (ϵ 2 × 10^4). Anal. (C$_{12}$H$_9$N$_3$) C, H, N.

Powdered sodium amalgam (5%, 240 g) was added in portions over 15–20 min at room temperature to a vigorously stirred solution of **23** (6.7 g, 0.034 mol) in a mixture of MeOH (360 mL) and H$_2$O (120 mL) through which CO$_2$ was passed. After addition, CO$_2$ was passed through the stirred mixture for a further 2.5 h, the solution was decanted and filtered, and the residue was washed with MeOH. Evaporation and addition of H$_2$O gave **24** (5 g, 76%), mp 105–107 °C. Recrystallization from EtOH/H$_2$O gave a sample: mp 108.5–110 °C; IR ν_{CN} 2237 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 3.25 (br, CH$_2$), 4.2 (br, CH), 7.25 (m, Ph) (imidazole protons not observed due to the presence of paramagnetic impurities). Anal. (C$_{12}$H$_{11}$N$_3$) C, H, N.

β-Benzylhistamine (25). Method A. A solution of **24** (1 g 0.005 mol) in dry THF (60 mL) was added dropwise to a stirred suspension of LiAlH$_4$ (0.77 g) in THF (20 mL), and the mixture was heated under reflux for 2 h. Addition of H$_2$O, followed by filtration and evaporation, gave an oily base, which was converted to the dimaleate on addition of a solution of maleic acid in MeOH. Addition of ether gave an oily solid, which was recrystallized from i-PrOH–Et$_2$O to give the dimaleate of **25** (0.76 g, 35%): mp

146–148 °C; ^{1}H NMR (D$_2$O) δ 3.15 (m, CH$_2$Ph), 3.55 (m, CH$_2$N + CH), 6.38 (s, maleate), 7.30 (m, Ph + Im 4 H), 8.71 (d, Im 2 H). Anal. (C$_{20}$H$_{23}$N$_3$O$_8$) C, H, N.

Method B. A solution of BF$_3$·Et$_2$O (5.7 g) in dry diglyme (20 mL) was added dropwise over 30 min to an ice-cooled mixture of NaBH$_4$ (1.3 g, 0.03 mol) and a solution of **24** (1 g, 0.005 mol) in diglyme (20 mL). The mixture was stirred for 3 h and left to stand overnight, and the diglyme was removed at 40 °C (0.5 mm). The residue (in MeOH) was acidified with 11 N HCl and then basified with aqueous KOH, and the mixture was heated under reflux for 30 min. The mixture was acidified (11 N HCl) and evaporated, the residue was extracted, and the aqueous solution was extracted with CHCl$_3$. Evaporation and addition of maleic acid gave the dimaleate of **25** (0.6 g, 27%), mp 145–147 °C.

N^α-Benzylhistamine (27). Reduction of N^α-benzoylhistamine[20] (2 g, 0.0093 mol) with LiAlH$_4$ (0.35 g, 0.0093 mol) in dry THF, followed by treatment with EtOH–HCl and recrystallization from EtOH–Et$_2$O, gave the dihydrochloride of **27** (1.7 g, 67%), mp 225–227 °C (lit.[10] mp 220–222 °C). HPLC analysis of **27** on a Partisil SCX column, with 0.125 M (NH$_4$)H$_2$PO$_4$ as the mobile phase, showed a small impurity (0.6% M) with a retention time corresponding to that of histamine.

Alkylation of the Prosthetic Heme in Cytochrome P-450 during Oxidative Metabolism of the Sedative–Hypnotic Ethchlorvynol

Paul R. Ortiz de Montellano,* Hal S. Beilan, and James M. Mathews

Departments of Pharmaceutical Chemistry and Pharmacology, University of California, San Francisco, California 94143. Received January 25, 1982

The clinically used sedative–hypnotic ethchlorvynol destroys hepatic microsomal cytochrome P-450 enzymes in a process catalyzed by the same hemoproteins. Abnormal porphyrins accumulate in the livers of phenobarbital-pretreated rats after administration of ethchlorvynol. The abnormal porphyrin fraction has been isolated and shown to consist of the four possible regioisomers of N-(5-chloro-3-ethyl-3-hydroxy-2-oxo-4-pentenyl)protoporphyrin IX. Cytochrome P-450 inactivation thus appears to result from alkylation of the prosthetic heme by the oxidatively activated acetylenic function in ethchlorvynol. The autocatalytic destruction of the hemoprotein is likely to alter the metabolism and elimination of ethchlorvynol and coadministered drugs and may be the cause of the porphyrinogenic properties of ethchlorvynol.

Ethchlorvynol (1-chloro-3-ethyl-1-penten-4-yn-3-ol), introduced in 1955, is still in use as a sedative–hypnotic, although its utility has been diminished by evidence that it is a habituating agent with a lower safety margin than the short-acting barbiturates.[1] Its continued use and abuse, however, remain sufficiently widespread for ethchlorvynol to be implicated in a significant number of drug overdose incidents.[2] Surprisingly little is known, considering the 25 years it has been in clinical use, concerning the metabolism of ethchlorvynol or its interactions with hepatic enzymes. Although little unchanged drug is excreted,[3] only a minor fraction of the administered drug is accounted for by the known metabolites. The only metabolites that have been clearly identified are an unusual

C-glucuronide of ethchlorvynol[4] and 1-chloro-3-ethynyl-1-pentene-3,4-diol,[5] the derivative in which the ethyl group has been hydroxylated. The disappearance of ethchlorvynol from plasma is first order at therapeutic doses[3] but appears to be zero order in overdose situations,[6] a change which suggests that elevated serum concentrations of the drug saturate the processes responsible for its removal. The pertinent observation has also been made that heme biosynthesis is stimulated in vitro[7] and in vivo[8] by ethchlorvynol, an interaction that may explain its deleterious effect in patients with acute porphyrias.

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Table I. Destruction of Hepatic Microsomal Cytochrome P-450 from Phenobarbital-Pretreated Rats by Ethchlorvynol

incubation	% cytochrome P-450 loss[a]			P-450 loss,[b] nmol	heme loss,[b] nmol
	10 min	20 min	30 min		
complete	30 ± 3	35 ± 3	38 ± 2	0.52 ± 0.02	0.32 ± 0.03
−NADPH	ND	ND	ND		
−O$_2$		4 ± 2			
−ethchlorvynol	ND	ND	ND		

[a] The incubation conditions are described under Experimental Section.   Averages of three determinations ± SD are given in the table; ND = not detectable.   [b] Correlation of P-450 and heme loss was done after a 20-min incubation in a separate experiment.   The ratio of P-450 to heme loss varied with time of incubation; the values given here are representative.

Recent work has established that rat hepatic cytochrome P-450 is vulnerable to self-catalyzed inactivation during the metabolism of unsaturated substrates as simple as ethylene[9,10] and acetylene.[11,12]   The destruction of cytochrome P-450 by vinyl chloride[13,14] and vinyl fluoride[15] has also been recorded.   The destruction of cytochrome P-450 by ethylene reflects alkylation of the prosthetic heme moiety by the oxidatively activated substrate, N-(2-hydroxyethyl)protoporphyrin IX being isolated after removal of the iron atom.[10]   The destruction of the enzyme by acetylene and vinyl fluoride results in covalent attachment to a heme nitrogen of a 2-oxoethyl moiety.[15]   These and related results have led us to postulate that the potential for prosthetic heme alkylation is inherent in the oxidative metabolism of terminal isolated π bonds, although this potential can be suppressed by substrate structural features.   A corollary of this postulate is that drugs with terminal unsaturated bonds may destroy cytochrome P-450 and thus alter their own metabolism or that of coadministered agents.   In a recent study we have clearly demonstrated that 2-isopropyl-4-pentenamide, a known cytochrome P-450 destructive agent, markedly alters the metabolism of propranolol in dogs.[16]   We now report that ethchlorvynol, by virtue of its terminal acetylene group, effectively destroys hepatic cytochrome P-450.

Results

Incubation of ethchlorvynol (1 mM) with hepatic microsomes from phenobarbital-pretreated rats in the presence of both oxygen and NADPH resulted in time dependent loss of spectroscopically detectable cytochrome P-450 (Table I).   The loss of cytochrome P-450 appeared to level off after 20–30 min, at which time approximately 40% of the enzyme had been lost.   Essentially no enzyme was lost if either NADPH or ethchlorvynol was omitted from the incubation mixture.   Enzyme loss was also drastically reduced if the oxygen concentration was reduced by bubbling argon through the mixture prior to initiating the incubation by addition of NADPH.   Measurements of the heme concentration before and after
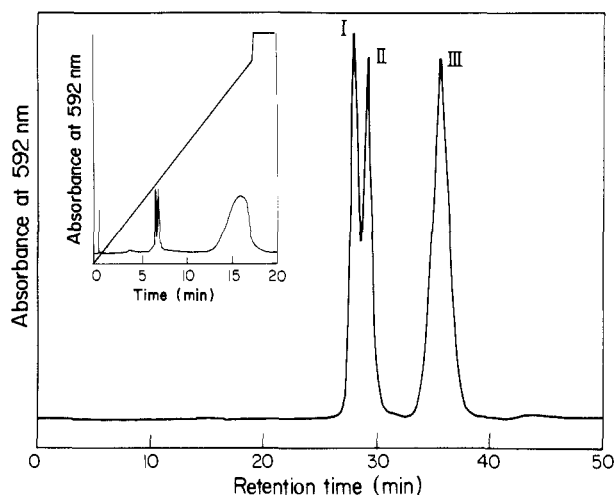
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Figure 1. High-pressure liquid chromatographic separation of the abnormal hepatic porphyrin isomers.   The zinc-complexed porphyrin fraction was purified by high-pressure liquid chromatography on a Partisil 10-PAC (9.4 × 250 mm) column (inset).   The 20-min linear gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran is indicated by the diagonal line.   The two sharp peaks at 14–16 min are protoporphyrin IX and another contaminant; the broad peak is due to the zinc-complexed porphyrin isomers.   The broad band, after demetallation, was chromatographed on a Partisil 10-PAC column (9.4 × 500 mm) eluted with 12:12:1 hexane/tetrahydrofuran/methanol.   Three peaks, numbered in order of elution, were obtained (main figure).   The ratio of the peak areas, in order of elution, is approximately 1:1:1.9.

incubation for 20 min with ethchlorvynol (Table I) show that the heme content decreases in parallel with cytochrome P-450, although the data suggest that the enzyme is lost more rapidly than microsomal heme.

The finding that ethchlorvynol destroys cytochrome P-450 and heme in vitro is confirmed, and the mechanism of at least part of the destructive process clarified, by the isolation of abnormal heme degradation products from the livers of phenobarbital-pretreated rats injected with ethchlorvynol (100 mg/kg).   The abnormal porphyrin fraction, a broad peak when subjected to high-pressure liquid chromatography in the zinc-complexed form, is resolved into three distinct peaks when rechromatographed after removal of the zinc ion (Figure 1).   The third peak is slightly broader and less symmetric than the first two peaks.   The peaks have been designated, in order of elution from the column, as isomers I–III.   In a qualitative sense, ethchlorvynol is among the agents that provide the highest yields of abnormal hepatic porphyrins.   Assuming an extinction coefficient of 125 000 M^{-1}, the isolated yield of the combined porphyrin isomers ranged from 30 to 85 μg/rat.

The structures of the porphyrin isomers have been elucidated by spectroscopic methods.   The electronic absorption spectra of the three chromatographic fractions in the metal-free form are indistinguishable.   An etio-type spectrum with a Soret band at 416 nm and absorption maxima at 511, 545, 592, and 648 nm (all values ± 1 nm)
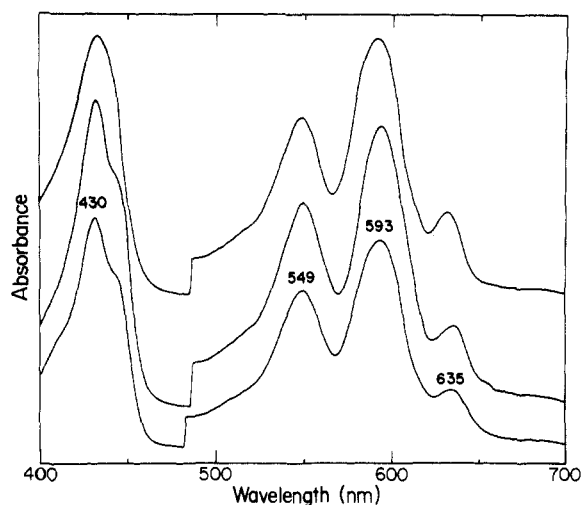
Figure 2. Electronic absorption spectra of the zinc-complexed abnormal porphyrin isomers: isomer I (bottom), isomer II (middle), isomer(s) III (top). See Figure 1 for identification of the isomer fractions. The Soret bands are recorded at a 10× higher attenuation than the rest of the spectra.

Figure 3. NMR spectrum (360 MHz) of the zinc complex of isomer I. The regions from 1 to 2 ppm and from −1 to −4 ppm are not shown but are devoid of peaks, except for a water peak at 1.5 ppm and an impurity peak at 1.2 ppm. The peak due to $CHCl_3$ at 7.21 ppm is shown truncated. An expanded scale reproduction of the 3 to 5-ppm region is shown in the bottom tracing of the inset. The upper tracing of the inset shows the collapse of a doublet at 3.24 ppm to a singlet (arrow) on irradiation of the protons at 4.98 ppm.

is obtained in each instance (not shown). Very similar values characterize the spectra of abnormal porphyrins obtained with other olefins[9] and acetylenes.[12] The spectra of the porphyrin isomers after complexation with divalent zinc are also similar to each other and to those of other abnormal porphyrins (Figure 2). An important difference, however, is the presence of a long-wavelength shoulder on the Soret band of isomers I and II but not of isomer(s) III. We have previously observed with synthetic N-alkylated protoporphyrin IX isomers that the shoulder is present in isomers with the N-alkyl group on the vinyl-substituted pyrrole rings (rings A and B) but not in those with the N-alkyl group on pyrrole rings C and D.[20,22]

Field-desorption mass spectrometric analysis of each of the metal-free porphyrin isomers reveals that each one exhibits a monoprotonated molecular ion at m/e 751 and a smaller ion, as expected if one chlorine is present, at m/e 753. There are no significant fragmentation peaks in these spectra. The mass spectrum of the mixture of isomers after complexation with divalent zinc has also been determined. This exhibits a monoprotonated molecular ion cluster with the strongest peak at m/e 815, as expected for a substance of molecular weight 750 complexed with the principal zinc isotope (M_r 64). Subtraction of the molecular weight of the dimethyl ester of protoporphyrin IX (M_r 590) (the ester is expected because of the workup procedure) suggests the incorporation of the equivalents of ethchlorvynol (M_r 144) and an oxygen atom into each of the isomers.

NMR studies confirm that each of the isomers is a dimethyl esterified protoporphyrin IX with an N-alkyl group derived from the substrate plus an oxygen atom. The spectrum of isomer I is reproduced in Figure 3 (see paragraph at end of paper concerning Supplementary Material). The signals in the spectra of the three isomers can be assigned as follows: 10.1–10.5 (meso protons), 7.8–8.3 (internal proton on vinyl substituents), 6.1–6.5 (terminal protons on vinyl substituents), 4.7–5.2 (one substrate-derived vinyl proton), 4.0–4.5 (internal methylene protons on propionic acid side chains), 2.5–3.8 (porphyrin methyls, methoxys, external methylenes of propionic acid side chains, and the second substrate-derived vinyl proton), 0.7–0.9 (ethchlorvynol hydroxy), −0.4 to −1.0 (ethchlorvynol ethyl group), and −4.1 to −4.3 ppm (NCH₂CO). Integration of the signals gives results consistent with this distribution of protons. The assignments have been confirmed for
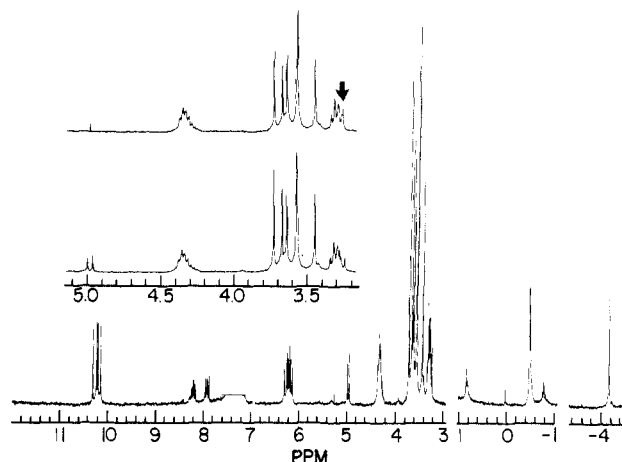
isomer I, the only one of the three subjected to detailed NMR analysis, by the following experiments. Although addition of deuterated water causes little change in the signals except for the water impurity peak at 1.5 ppm, the further addition of a trace of trifluoroacetic acid results in disappearance of the signal at −4.2 ppm (see paragraph at end of paper concerning Supplementary Material). As noted previously, porphyrin N-alkyl groups do not undergo proton exchange with the medium under these conditions unless they are activated by a vicinal carbonyl group.[15] The peaks attributed to the ethchlorvynol ethyl group are cleanly resolved after addition of trifluoroacetic acid into two discrete multiplets (0.2 and 0.1 ppm) due to the nonequivalent methylene protons and a multiplet at −1.8 ppm due to the methyl protons. The indicated relationship between these protons is verified by the observation that the methylene proton multiplets collapse to doublets when the methyl protons are irradiated (see paragraph at end of paper concerning Supplementary Material). Irradiation of the chlorovinyl proton at 4.98 ppm has been used to locate the vinyl proton coupled to it. This decoupling experiment (Figure 3, inset) shows that the other substrate-derived vinyl proton appears as a doublet at 3.24 ppm. All of the protons associated with the ethchlorvynol substructure have thus been identified. The proton assignments for the porphyrin framework are based on confirmed assignments of the same protons in other N-alkylated protoporphyrin IX derivatives.[19-22] The isolated porphyrin is unambiguously identified by these results as one isomer of dimethyl esterified N-(5-chloro-3-ethyl-3-hydroxy-2-oxo-4-pentenyl)protoporphyrin IX (Figure 4).

The similarities in the NMR and other spectra of isomers II and III with those of isomer I establish that all three porphyrins have the same basic structure, except that it is evident from the NMR spectrum of fraction III that

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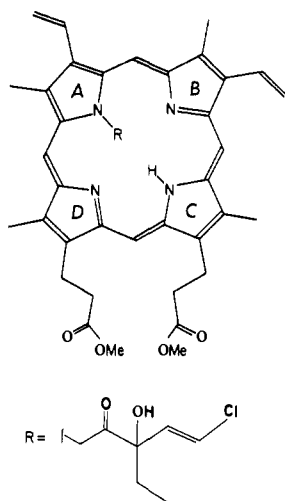
Figure 4. Structure of the porphyrin adducts obtained with ethchlorvynol. The structure of isomer I is shown. Isomer II is similar except that the *N*-alkyl group is on the nitrogen of pyrrole ring B. Isomer III consists of a mixture of analogous structures with the *N*-alkyl group on pyrrole rings C and D. The absolute stereochemistry at the chiral center is not known.

Table II. Chemical Shifts, Relaxation Times, and Nuclear Overhauser Effects for Isomer I

group identity	chem shift, ppm	T_1, s	meso position exhibiting NOE
α meso	10.313	0.90	
β meso	10.237	0.86	
δ meso	10.207	0.80	
γ meso	10.152	0.47	
methylenes vicinal to ring	4.314		γ
methoxy	3.708	1.35	none
methoxy	3.652	1.39	none
3-methyl[a]	3.620	0.65	α
5- and 8-methyls	3.552	0.66	β and δ
1-methyl[b]	3.429	0.61	δ

[a] Irradiation of 3-methyl protons causes sharpening of low-field (8.206 ppm) internal vinyl multiplet. [b] Irradiation of 1-methyl protons causes sharpening of high-field (7.919 ppm) internal vinyl multiplet.

it is a mixture of two or more related isomers. Direct evidence for the presence of a mixture is provided by the multiplicity of ethchlorvynol methyls at ~4.2 ppm, of meso protons at 10.2–10.5 ppm, and of chlorovinyl doublets at 4.8–5.2 ppm (see paragraph at end of paper concerning Supplementary Material). Decoupling studies (not shown) have established that the second chlorovinyl group proton in isomer II is at 3.22 ppm and that the chlorovinyl proton doublets at 4.8–5.2 ppm in the fraction III mixture are not coupled to each other but to protons also located in the 3.2–3.6 ppm region.

If the possibility of diastereomeric products introduced by the presence of a chiral center in ethchlorvynol is momentarily ignored, four regioisomers of the porphyrin–ethchlorvynol adduct are possible. These isomers derive from alkylation of the four nonequivalent nitrogens in protoporphyrin IX. Isomers I and II are identified as structures with the *N*-alkyl group on the vinyl-substituted pyrrole rings not only because of the shoulder on the Soret band in their spectra (Figure 2) but also because of the presence of two distinct multiplets for the internal protons of the vinyl substituents (Figure 3).[20,22] Conversely, the mixture of isomers in fraction III must be N-alkylated on the propionic acid substituted rings because there is no shoulder on their Soret band and only one multiplet for the internal vinyl protons, but a distinct multiplet for each of the methylene groups in the two propionic acid side chains (at 4.35, 4.15, 3.20, and 2.75 ppm).[20,22]

The question of whether pyrrole ring A or B is N-alkylated in isomer I has been addressed through an NMR protocol validated in earlier work with synthetic and biologically obtained porphyrins.[20,21] Each of the signals in the spectrum must first be assigned to a specific proton or set of protons through the combined use of (a) proton relaxation times (T_1), (b) nuclear Overhauser effects (NOE) between the meso protons and the vicinal methyl groups, and (c) long distance decoupling of the internal vinyl protons from the vicinal methyl protons. In the case of a ring A or B alkylated isomer, the N-alkylated ring is that which bears the vinyl group with the internal vinyl proton at highest field. The γ-meso proton and the ester methoxy groups in isomer I are readily identified by, respectively, their abnormally low and high T_1 values (Table II). The γ-meso proton assignment is confirmed by the NOE ob-

served on irradiation of the propionic side-chain methylene protons. The δ-meso proton signal is identified by the fact that it is the only one that exhibits a NOE on irradiation of two different methyl groups. The difficult differentiation of the α- and β-meso proton signals is made possible by the fact that irradiation of one of the methyls that exhibits a NOE with the δ-meso proton results in sharpening of the high-field internal vinyl proton signal. The methyl in question must therefore be that at position 1. The 3-methyl group is similarly located because its irradiation causes a sharpening of the low field internal vinyl proton signal. The NOE between the 3-methyl and the α-meso proton uniquely identifies the latter. The resulting complete assignments are summarized in Table II. In view of the fact that the 1-methyl and the internal proton on the 2-vinyl group are at high field, the N-alkyl group in isomer I must be on pyrrole ring A (Figure 4). The alkylated nitrogen in isomer II, by exclusion, is therefore that of pyrrole ring B.

The methylene protons vicinal to the chiral center in the N-alkyl group of isomer I appear as two 1-proton multiplets (Supplementary Material). Two interpretations are possible for this signal pattern. One is that the two protons of the methylene group, which are not equivalent, give rise to distinct signals. Collapse of the two multiplets to two doublets on irradiation of the vicinal methyl is then expected because the two protons would still be coupled to each other. The second interpretation is that heme alkylation occurs equally well with both enantiomers of ethchlorvynol and that the methylene protons of the resulting diastereomers appear at different positions in the NMR spectrum. Each of the methylene proton multiplets in the spectrum of isomer I would then correspond to the protons in one diastereomer. The collapse of each multiplet to a two-peak signal on irradiation of the methyl is consistent with this view if the two protons in each methylene resonate at slightly different frequencies, in which case each peak in the decoupled spectrum corresponds to a single proton. This latter circumstance is to be expected because the two methylene protons in ethchlorvynol itself are sufficiently nonequivalent to appear as a multiplet rather than as a quartet (not shown). A distinction between these alternatives, however, is provided by the observation that the multiplet at ~0.78 ppm (Figure 3) collapses to a *singlet* when the superimposed methyl and methylene protons at ~0.5 ppm are irradiated (not shown). These results, consistent only with the first explanation, lead to the conclusion that isomer I results from the reaction of heme with only one of the two ethchlor-

vynol enantiomers or, if reaction occurs with both, that the resulting diastereomeric adducts exhibit identical NMR spectra. The stereoisomeric composition of isomers II and III has not been examined in detail. The minor extraneous peaks in the NMR spectrum of isomer II, however, are most probably associated with a trace contamination by isomer I (note that the peaks were not completely resolved by chromatography; Figure 1). The possibility that isomer II is a diastereomer of isomer I is very unlikely because the differences in their NMR spectra are associated exclusively with protons of the porphyrin framework rather than the ethchlorvynol substructure. Little can be said concerning the presence of stereoisomers in fraction III because of the mixture of isomers in the sample. The regio- and stereochemistry of prosthetic heme alkylation is the subject of a continuing investigation.

Discussion and Conclusions

The destruction of cytochrome P-450 by ethchlorvynol is time, NADPH, and O_2 dependent and is accompanied by loss of microsomal heme (Table I). The loss of heme is due, as shown by in vivo studies, to the formation of adducts between ethchlorvynol and (presumably) the prosthetic heme of cytochrome P-450. The porphyrin products derived from the ethchlorvynol–heme adducts have been isolated and shown to be isomers of N-(5-chloro-3-ethyl-3-hydroxy-2-oxo-4-pentenyl)protoporphyrin IX (Figure 4). The isolated porphyrins provide direct evidence for a reaction in which a heme nitrogen is alkylated by the terminal carbon of the ethynyl group in the substrate after this moiety has been oxidatively activated. The destruction of cytochrome P-450 by ethchlorvynol thus occurs by a mechanism similar to that which governs the destruction of the enzyme by propyne,[21] acetylene,[15] and probably other acetylenes.[11,12] We have proposed that this mechanism is initiated by catalytic transfer of an oxygen atom from cytochrome P-450 to the acetylenic π bond,[12,15,21] a metabolic reaction for which we have provided independent evidence.[23,24] The product of triple bond oxidation can be formulated as an unsaturated epoxide, an α-oxocarbene, or an acyclic dipolar species, all of which are interconvertible, although the equivalent radical species can also be envisioned if oxygen transfer is a single-electron process. The unstable intermediate, whatever its detailed electronic structure, in turn reacts with the prosthetic heme nitrogen atom to give the N-alkylated derivative from which the isolated porphyrins are obtained by removal of the iron atom. The details of the alkylation sequence, including, for example, whether a carbene–iron complex precedes N-alkylation, nevertheless remain to be elucidated.

The destruction of cytochrome P-450 by ethchlorvynol may have significant pharmacological and toxicological consequences. If human hepatic cytochrome P-450 isozymes are similarly destroyed, their destruction will be reflected in the metabolic profile and rate of elimination both of ethchlorvynol and of coadministered drugs. The practical significance of this metabolic impairment will depend on the importance of the vulnerable isozymes in the metabolism of the drugs in question and on the reserve capacity of alternative metabolic pathways to compensate for the compromised oxidative route. The evidence for a change from first order to zero order elimination kinetics as one passes from therapeutic to toxic doses of eth-

chlorvynol[6] suggests that, at least in the overdose situation, the loss of cytochrome P-450 may significantly decrease the rate of ethchlorvynol elimination. Our recent demonstration of altered propranolol metabolism in a dog treated with a cytochrome P-450 destructive agent[16] also recommends that ethchlorvynol be used in multiple drug treatments with caution, although little data are available on metabolic interactions of ethchlorvynol with other drugs. Finally, the destruction of cytochrome P-450 by ethchlorvynol may be responsible, at least in part, for the increase in heme synthesis associated with this drug and for its contraindication in porphyric patients.[7,8] Accelerated heme synthesis could result from derepression of the pathway due to a decrease in the regulatory heme pool mediated by sequential prosthetic heme destruction and replacement.[25] The prosthetic heme adduct may alternatively, like N-methylprotoporphyrin IX,[26-28] inhibit ferrochelatase. Although some prosthetic heme adducts have been reported not to inhibit ferrochelatase,[27] we have recently shown that the inhibitory activity depends on which nitrogen is alkylated.[29] The activity of isomers with the N-alkyl group on pyrrole ring A or B was found to be relatively less dependent on the size of the N-alkyl group.[29] The formation of these isomers with ethchlorvynol thus makes ferrochelatase inhibition a possibility.

Experimental Section

Ethchlorvynol was obtained by extraction of commercial (Abbott Laboratories) 500-mg capsules with methylene chloride, followed by an aqueous wash of the extract, drying over anhydrous sodium sulfate, and distillation (bp 64–66 °C at 5 mmHg). NMR analysis indicated that the double bond was exclusively in the E configuration. All other materials and reagents were commercial products of the highest available purity.

Microsomes from phenobarbital-pretreated Sprague–Dawley male rats were prepared as previously reported.[9,10] Protein concentrations were measured by the Lowry procedure with 1% serum albumin as the standard.[17] Incubations, carried out as previously described,[9,10] contained the following: microsomal protein (1 mg/mL), EDTA (1.5 mM), KCl (150 mM), NADPH (1 mM), and ethchlorvynol (1 mM), all in 0.1 mM Na–K phosphate buffer (pH 7.4). Omissions from this standard incubation mixture are cited where appropriate. Incubations under decreased oxygen tension were carried out after argon had been bubbled through the incubation mixture. Incubations were initiated by the addition of NADPH and were carried out at 37 °C. Heme concentrations were measured by the pyridine hemochromogen assay as reported previously.[18]

Hepatic green pigments were isolated by procedures analogous to those already reported.[10,12,19] In brief, ethchlorvynol (100 mg/kg dose) in polyethylene glycol 200 was administered ip to 250–300 g, Sprague–Dawley male rats that had been pretreated for 4 days with an 80 mg/kg daily intraperitoneal injection of sodium phenobarbital in water. The rats were decapitated 4 h after ethchlorvynol injection, and their livers were perfused in situ with cold isotonic saline before they were removed and homogenized in isotonic saline. The homogenate was allowed to stand overnight at 4 °C in 5% (v/v) H_2SO_4/methanol (100 mL/liver). The mixture was extracted with CH_2Cl_2, and the extracts were extensively washed with water before a small amount of zinc acetate in methanol was added. The solvent was removed after the extracts

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were dried (anhydrous sodium sulfate), and the residue obtained was subjected to thin-layer chromatography on 2000-μm silica gel G (Analtech) plates developed with 3:1 (v/v) $CHCl_3$/acetone containing 0.5% methanol. The green (red fluorescent) band was collected and further purified by high-pressure liquid chromatography on a Partisil 10-PAC column (9.4 × 250 mm) with a 20-min linear gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran. The demetallated porphyrin isomers obtained by treatment of the purified pigment with 5% H_2SO_4 in methanol[9,12] were then separated and further purified by high-pressure liquid chromatography on a Partisil 10-PAC column (9.4 × 500 mm) eluted with (v/v) 12:12:1 hexane/tetrahydrofuran/methanol. For NMR studies, zinc acetate in $CHCl_3$ was added to each of the isomers, and the resulting zinc complex of each isomer was finally purified by high-pressure liquid chromatography on a 4.6 × 250 mm Partisil 10-PAC column eluted with a 20-min linear gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran.

Cytochrome P-450 and heme assays were performed on an Aminco DW-2A spectrophotometer. Porphyrin electronic absorption spectra were recorded in CH_2Cl_2 on a Varian Cary 118 instrument. Field-desorption mass spectra were obtained as previously described[12] on a modified Kratos AEI MS-9 instrument. NMR studies were performed on a 360-MHz Nicolet NT-360

FT-NMR instrument. The $CHCl_3$ peak at 7.21 ppm was used as an internal reference. Conditions for nuclear Overhauser effect (NOE) and relaxation time measurements have been reported.[20,21] NMR samples were prewashed with NaCl solution to ensure the presence of chloride as the zinc counterion. The deuterated $CHCl_3$ used in the NMR work was stored over K_2CO_3 to eliminate acidic products.

Supplementary Material Available: NMR spectra of isomers II and III (Figure 5) and deuterium exchange and decoupling experiments with isomer I (Figure 6) (2 pages). Ordering information is given on any current masthead page.

Species- or Isozyme-Specific Enzyme Inhibitors. 8.[1] Synthesis of Disubstituted Two-Substrate Condensation Products as Inhibitors of Rat Adenylate Kinases

Francis Kappler, Ton T. Hai, Masanobu Abo, and Alexander Hampton*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. Received March 22, 1982

Syntheses are described of 5'(R)- and 5'(S)-C-Me-ATP, 5'(R)- and 5'(S)-C-n-Pr-ATP, and the phosphonate isostere of ATP with a C(5')-CH_2-P system. Determinations of K_M (ATP)/K_i for competitive inhibition showed that two of the five compounds inhibited rat muscle adenylate kinase (AK-M) 8–9.5 times more effectively than AK II (present in poorly differentiated rat hepatoma tissue) and that two other compounds inhibited AK II at least 2-fold more effectively than AK-M, further indicating that monosubstituted substrate derivatives are potentially useful probes in the design of isozyme-selective inhibitors. P^1-[8-(Ethylthio)adenosine-5']-P^5-(adenosine-5') pentaphosphate (8-SEt-Ap_5A) is a potent dual substrate site inhibitor of the rat AK isozymes with selectivity for AK II. Three derivatives of 8-SEt-Ap_5A were synthesized: P^1-[8-(ethylthio)adenosine-5']-P^5-[5'(R)-C-methyladenosine-5'] pentaphosphate (I), its 5'(R)-C-n-Pr analogue (II), and di(8-SEt)-Ap_5A (III). Unsymmetrical pentaphosphates, such as I and II, are shown to be readily accessible via reaction of a derivative of ATP γ-piperidinate with an ADP derivative. Conversion of 8-SEt-ATP to 8-(ethylthio)adenosine 5'-trimetaphosphate, followed by reaction of the latter in situ with added piperidine, gave 8-SEt-ATP γ-piperidinate quantitatively. Except in the interaction of III with AK-M, I–III acted as two-site competitive inhibitors of AK-M and AK II with K_i < K_M of AMP or ATP. Inhibitory potencies [K_M (ATP)/K_i] of I–III with the two isozymes varied over more than a 95-fold range, and inhibitory potencies for AK-M relative to those of AK II varied more than 61-fold. III was an effective inhibitor of AK II (K_M/K_i = 8 and 14 with AMP and ATP, respectively) and exhibited at least 4 times more selectivity for AK II [relative inhibitory potency, AK II/AK-M, >22] than 8-SEt-Ap_5A.

Evidence, summarized previously,[2] suggests that selective inhibitors of fetal-type isozymes of key metabolic enzymes could, if available, be potentially useful in the design of new types of antineoplastic agents. Studies of approaches that might have utility in the design of isozyme-selective inhibitors showed that such inhibitors were frequently produced when a single short (1 to 3 atoms) substituent was introduced at various atoms in turn of a substrate of the target enzyme. This result was obtained with each of three enzymic substrates studied.[1,3–6] The

selective inhibitors so obtained were usually of weak or moderate potency, however. In the case of one target enzyme, adenylate kinase (AK), it proved possible to maintain the selectivity while enhancing potency 1000-fold by elaborating a monosubstituted substrate derivative into a monosubstituted two-substrate condensation product that appears to bind simultaneously to two adjoining substrate sites.[6] In the present study, two-substrate condensation products bearing a substituent at each substrate moiety were synthesized in order to further explore the potential of dual-site inhibitors for isozyme selectivity and with the hope of progressing further toward a potent and specific inhibitor of the isozyme AK II that predominates

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