THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 12, Issue of June 25, pp. 5578–5585, 1980
Printed in U.S.A.

Self-catalyzed Inactivation of Hepatic Cytochrome P-450 by Ethynyl Substrates*

(Received for publication, November 26, 1979, and in revised form, February 20, 1980)

Paul R. Ortiz de Montellano‡ and Kent L. Kunze

From the Department of Pharmaceutical Chemistry, School of Pharmacy, and Liver Center, University of California, San Francisco, California 94143

The following acetylenic substrates have been shown to mediate NADPH-dependent loss of cytochrome P-450 on incubation with hepatic microsomes from phenobarbital-pretreated rats: 1-ethynylcyclohexanol, 1-ethynylcyclopentanol, 3-methyl-1-pentyn-3-ol, norethisterone, (1-methoxycyclohexyl)acetylene, 3-(2,4-dichlorophenoxy)-1-propyne, 3-(4-nitrophenoxy)-1-propyne, 3-phenoxy-1-propyne, 4-phenyl-1-butyne, 3-phenyl-1-propyne, cyclohexylacetylene, acetylene, 3-pentyn-2-ol, 4-methyl-2-octyn-4-ol, 2-hexyne, phenylacetylene, and *N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide. A 10-fold higher nominal concentration of the last two agents was required to obtain the same degree of enzyme loss observed with the other agents at a 1 mM concentration. *In vivo* administration of acetylene gas and nine of the monosubstituted acetylenes led to accumulation of abnormal hepatic pigments. Similar pigments were not observed in rats treated with disubstituted acetylenes. The pigments obtained with acetylene gas, norethisterone, and six other substrates, after isolation, methylation, and purification, exhibited essentially identical electronic absorption spectra. Field desorption mass spectrometric analysis of these eight pigments has established that each one gives a molecular ion which corresponds to the sum of the molecular weights of the dimethyl ester of protoporphyrin IX plus the substrate plus (probably) an oxygen atom. These results are used to formulate a destructive mechanism in which the enzyme prosthetic heme covalently binds to the substrate during attempted metabolism of the triple bond.

Cytochrome P-450[1] monooxygenases, a diverse group of enzymes related by similarities in their catalytic function and by the presence of a heme prosthetic group, dominate the hepatic metabolism of xenobiotic lipophilic substances (1–3). Each of these enzymes catalyzes reductive cleavage of molecular oxygen with concomitant transfer of 1 of the oxygen atoms to the substrate (1–3), although the observable consequences of oxygen transfer depend on the precise nature of the substrate. In general, the established immediate outcome of cytochrome P-450-catalyzed oxygen transfer is (*a*) formation of a hydroxyl derivative by insertion into the bond between a hydrogen and a heavier atom; (*b*) formation of an epoxide by addition across a carbon-carbon double bond; or (*c*) formation of a dipolar oxide by combination with the free electron pair of a heteroatom (4, 5). These primary reactions, however, often yield unstable structures which undergo secondary chemical transformations to give final experimentally observed products (4, 5). The rearrangement of enzymatically formed aryl epoxides to phenolic derivatives is an example of such a secondary process (6).

Although cytochrome P-450 enzymes involved in physiological biosynthetic pathways usually exhibit high substrate specificity, those forms of the enzyme significantly engaged in xenobiotic metabolism are characterized by broad and overlapping substrate selectivities (7–10). The effectiveness of this metabolic apparatus, which copes with the wide range of substances to which an individual is environmentally exposed, is optimized through differential induction of cytochrome P-450 isozymes by lipophilic substrates (10, 11). However, the broad specificity and differential inducibility of these monooxygenase enzymes render alterations in metabolism of one agent by another a relatively common phenomenon.

Inhibition of the metabolism of one substance by another in order to enhance pharmacological activity has been commercially exploited with the advent of insecticide synergists (12, 13). An impressive fraction of the substances shown to potentiate the action of insecticides is characterized by the presence of a monosubstituted acetylenic function. Among these active agents are aryl propargyl ethers (14–16), *N*-alkynyl phthalimides (17), alkynyl phosphate esters (12, 18), and alkynyl oxime ethers (12, 19). The mechanism by which these acetylenic substances alter insecticide metabolism and toxicity, however, has remained undefined except for the observation that *in vivo* administration of 3-(2,4,5-trichlorophenoxy)-1-propyne and 2-methylpropyl 2-propynyl phenylphosphonate to mice causes a measurable decrease in hepatic cytochrome P-450 content (18).

Research in a different biological arena has also provided evidence for altered drug metabolism due, in part, to the interaction of acetylenic groups with cytochrome P-450 enzymes. Synthetic 17-ethynyl sterols, hormonally active constituents of most birth control pills (20), have been found to inhibit oxidative drug metabolism (21–25). White and Müller-Eberhard (26) have reported that, at relatively high doses, ethynyl sterols specifically reduce hepatic cytochrome P-450 and heme levels in rats. Norethisterone and ethynylestradiol, the two ethynylsterols examined, selectively affected the phenobarbital-inducible isozymes (27) of cytochrome P-450. The

* This work has been supported by National Institutes of Health Grants GM-25515 and P-50 AM-18520. The Berkeley Biomedical and Environmental Mass Spectrometry Resource, where mass spectra were obtained, is supported by National Institutes of Health Grant RR 00719. The Computer Graphics Laboratory, used in preparation of Scheme 2, is supported by National Institutes of Health Grant RR 1081-021. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A Research Fellow of the Alfred P. Sloan Foundation.

[1] Cytochrome P-450 generally refers in this paper to the isozymes induced by phenobarbital, but occasionally is used in a generic sense to denote the class of hemoproteins whose reduced carbon monoxide spectra exhibit a maxima at around 450 nm.

additional and highly significant observation was made that cytochrome P-450 loss was accompanied by accumulation of a hepatic "green pigment" not unlike that found by other investigators in rats treated with 2-isopropyl-4-pentenamide (28–30).

Work in this laboratory has established that 2-isopropyl-4-pentenamide inactivates cytochrome P-450 by covalently binding to the heme prosthetic group during catalytic interaction with the enzyme (31–33). In a recent brief communication, we have also reported that radiolabeled norethisterone is covalently incorporated into the "green pigment" produced by treatment of rats with this sterol (34). Covalent attachment of the sterol to prosthetic heme, suggested by the radioisotopic data, was confirmed by the mass spectrometric molecular ion of the purified pigment, although the observed molecular ion could not be attributed to a specific molecular species (34). We now report an in-depth study of the interaction of acetylenes with cytochrome P-450 which demonstrates the generality of the process, unequivocally establishes the formation of substrate-heme adducts, and provides the basis for formulation of a tentative reaction mechanism. Aspects of this study have been presented at several meetings (35–37).

EXPERIMENTAL PROCEDURES

Substrates and Reagents—The following substances, of the highest grade available, were obtained from the indicated commercial source and were used without further purification: 1-ethynylcyclohexanol (**A**), 1-ethynylcyclopentanol (**B**), 3-methyl-1-pentyn-3-ol (**C**), 3-pentyn-2-ol (**O**), 4-methyl-2-octyn-4-ol (**P**), 4-phenyl-1-butyne (**I**), and 2-hexyne (**Q**) (Farchan Division, Story Chemical Corp.); cyclohexylacetylene (**L**) (Pfaltz-Bauer); phenylacetylene (**K**) (Aldrich Chemical Co.); and acetylene (**M**) (Matheson Chemical Co.). Literature procedures were used for the synthesis of (1-methoxycyclohexyl)acetylene (**E**) (38), 3-phenyl-1-propyne (**J**) (39), 3-phenoxy-1-propyne (**H**) (14), 3-(2,4-dichlorophenoxy)-1-propyne (**F**) (14), and 3-(4-nitrophenoxy)-1-propyne (**G**) (14). Norethisterone (17-hydroxy-19-nor-17-pregn-4-en-20-yn-3-one, **D**) was generously provided by Syntex Research, Palo Alto, Calif., and *N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide (**N**, code No. RH-315) by Rohm and Haas Co., Philadelphia, Pa. Solvents used for chromatographic purification of hepatic pigments were analytical grade and were distilled prior to use.

In Vitro Assay of Cytochrome P-450 Inactivation—The general procedure used, based on that of the Hoffmann-La Roche group (40), has been described (33). Incubation mixtures contained, in addition to substrates, the following: microsomal protein (1 mg/ml), NADPH (1 mM), KCl (150 mM), and EDTA (1.5 mM), all in 0.1 M phosphate buffer (pH 7.4). Acetylenic substrates, added without solvent at a nominal 1 mM concentration, were preincubated with the microsomal suspension for 10 min to allow substrate equilibration before NADPH was added to initiate the reaction. Nominal 10 mM concentrations of phenylacetylene and *N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide were used since these agents caused only a marginal loss of cytochrome P-450 at a 1 mM concentration. In all cases, control incubations were carried out in the absence of added substrates and, for each substrate, in the absence of NADPH. The loss of cytochrome P-450 in the absence of substrates was negligible, demonstrating that lipid peroxidation was effectively suppressed by the added EDTA.

Isolation of Abnormal Hepatic Pigments—Sprague-Dawley male rats weighing 200 to 250 g were injected intraperitoneally once a day for 4 days with an aqueous solution (80 mg/ml) of sodium phenobarbital (80 mg/kg dose). Acetylenic substrates were administered on the 5th day. Norethisterone (25 mg/kg) was injected intraperitoneally in 0.25 ml of trioctanoin after sonication of the mixture to disperse the sterol. All other solid agents (200 mg/kg) were injected intraperitoneally as a solution in 150 μl of ethanol. Liquid agents (50 μl/rat) were injected intraperitoneally without dilution in a solvent. Acetylene gas was administered by placing phenobarbital-pretreated rats for 4 h in a chamber through which a stream of air containing 10 to 15% (v/v) acetylene was passed at an approximate rate of 200 to 300 ml/min. Four hours after administration of acetylenic agents, rats were decapitated and their livers were perfused *in situ* with ice-cold 0.9% saline solution (100 ml/liver). The pooled livers of rats treated with a common agent were homogenized in 5% (v/v) H_2SO_4/methanol (4 ml/g of liver) and the homogenate was allowed to stand overnight at 4°C in the dark. All manipulations were carried out in the dark since the hepatic pigments are photosensitive. The homogenate was filtered to remove precipitated protein and equivalent volumes of water and chloroform were added. The brown chloroform layer was separated, washed with water until the washes were no longer acidic, and dried over anhydrous Na_2SO_4. After filtration, 2 ml of a 0.5% solution of zinc acetate in chloroform was added before solvent removal at a rotary evaporator. The preparation of zinc complexes at this stage prevents the formation of artifactual metal complexes during purification (32, 34). The oily brown residue obtained on solvent removal was chromatographed on preparative Silica Gel GF plates (Analtech, Inc.) using 3:1 chloroform/acetone as solvent. The zinc-complexed porphyrin pigments appear as visibly green, red-fluorescing bands with R_F values of 0.5 to 0.8. One or two such bands were observed, depending on the substrate (see text). The pigment-containing fractions were scraped from the plate and the pigment was extracted with acetone. The isolated zinc-complexed porphyrins were further purified by high pressure liquid chromatography on a Whatman Partisil 10-PAC column using methanol/tetrahydrofuran 4:1 (v/v) as solvent (2 to 3 ml/min of flow rate). The column effluent was monitored with a variable wavelength detector set at 431 nm. After removal of an aliquot of the collected pigments for spectroscopic analysis, the purified zinc complexes were demetalated by standing in 1 ml of 5% H_2SO_4/methanol for 15 min. Addition of equal volumes of water and chloroform, separation of the layers, washing of the chloroform fraction with water until the washes were no longer acidic, drying over anhydrous sodium sulfate, and solvent removal yielded pure, metal-free porphyrin pigments. The metal-free pigments were analyzed by high pressure liquid chromatography as previously described (31–34). In some instances, metal-free porphyrin pigments were reconverted into zinc complexes by the procedure given above.

Spectroscopic Analysis of Purified Pigments—The electronic (UV-visible) absorption spectra of both the metal-free and zinc-complexed pigments were determined in dilute chloroform solution on a Varian-Cary 118 spectrophotometer.

A Kratos/AEI MS-902 instrument with a field desorption source was utilized for the mass spectrometric studies. Operating conditions were the same as those previously reported (33). Except for norethisterone, a mass spectrum was obtained for each isolated porphyrin both in its free-base and zinc-complexed form. Due to the already high molecular weight of the norethisterone-porphyrin adduct, it was only possible to obtain a mass spectrum for the metal-free form. In those instances where pigments were resolved into two components, electronic absorption and mass spectrometric data were independently obtained for each of the two components.

In Vitro Pigment Formation—The formation of abnormal pigments *in vitro* was established in a larger scale incubation of norethisterone with the usual microsomal enzyme preparation. The incubation mixture already described was used except for the following alterations in concentration: norethisterone (1 mM), NADPH (4 mM), and microsomal protein (4 mg/ml). The total incubation volume was 40 ml and the incubation time 40 min. At the end of the incubation, the microsomal protein was sedimented by ultracentrifugation (100,000 × *g* for 1 h). The microsomal pellet was added to 5 ml of 5% H_2SO_4/methanol and was allowed to stand overnight. The pigment was extracted as already described. The electronic absorption spectra of the purified pigment, both as the free-base and as a zinc complex, were identical with those of the pigment obtained from *in vivo* experiments.

RESULTS

Sixteen acetylenic substrates have been incubated in this study with hepatic microsomes from phenobarbital-pretreated rats. A time-dependent decrease in measurable cytochrome P-450 content was caused by each of these substrates, in the presence of NADPH, under incubation conditions in which lipid peroxidation was suppressed (Fig. 1). No cytochrome P-450 was lost in the absence of added NADPH (data not shown). Although a quantitative structure-activity correlation is not possible due to the heterogeneous nature of the assay system and the differential solubility of the substrates, the data clearly show that all of the structures investigated exhibit the same order of magnitude activity except for phenylacetylene and *N*-(1,1-dimethyl-2-propynyl)-3,5-dichlorobenzamide. A 10-fold increase in concentration of these two substances

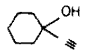
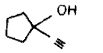
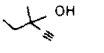
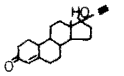
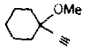
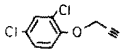
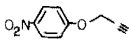
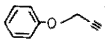
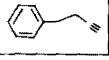
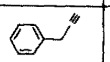
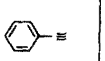
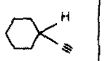
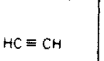
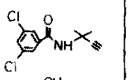
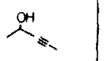
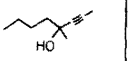
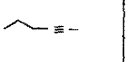
	SUBSTRATE	PERCENT LOSS OF CYTOCHROME P-450			IN VIVO PIGMENT
		10'	20'	30'	
A		18 ± 6	22 ± 6	26 ± 6	YES
B		18 ± 3	22 ± 4	29 ± 3	YES
C		17	24	34	—
D		20 ± 5	25 ± 2	27 ± 3	YES
E		22 ± 5	29 ± 6	30 ± 6	—
F		21 ± 6	23 ± 4	24 ± 3	YES
G		19 ± 10	23 ± 11	30 ± 11	YES
H		25 ± 3	31 ± 4	34 ± 2	YES
I		29 ± 3	49 ± 4	52 ± 3	YES
J		12 ± 6	24 ± 7	34 ± 1	YES
K		18 ± 3	25 ± 2	27 ± 2	YES
L		19 ± 2	23 ± 4	23 ± 4	—
M		24	33	40	YES
N		19 ± 5	30 ± 2	32 ± 3	—
O		24 ± 17	33 ± 10	35 ± 8	TOXIC
P		35 ± 4	45 ± 3	47 ± 1	NO
Q		27 ± 3	34 ± 5	36 ± 5	NO

FIG. 1. **Destruction of cytochrome P-450 on incubation of acetylenic substrates with hepatic microsomes from phenobarbital-pretreated rats.** Incubations were carried out as described under "Experimental Procedures." The data for norethisterone (**D**) are from Ref. 34 while the values for acetylene gas (**M**) are estimated from Ref. 41. The substrate concentration was nominally 1 mM except for phenylacetylene (**K**) and *N*-(1,1-dimethyl-2-propynyl)-3,5-dichlorobenzamide (**N**), which were examined at a 10 mM concentration since they caused only marginal cytochrome P-450 loss at the lower dose. The values given are averages of at least three determinations, with the exception that 3-methyl-1-pentyn-3-ol (**C**) was only assayed once. Standard deviations are given where applicable. Accumulation *in vivo* of an abnormal hepatic pigment in rats treated with the given agents (see "Experimental Procedures") is indicated by a yes in the column marked "pigment." A no in this column indicates that pigment was looked for but was not found; a *dash* indicates that the experiment was not performed. Incubation time is given in minutes.

was required to produce cytochrome P-450 losses equivalent to those observed with the other agents. Analogous data for acetylene gas, extrapolated from the graph reported by White (41), are included in the table for comparison.

Of the 17 active agents in Fig. 1, 13 were selected for *in vivo* investigation. The livers of phenobarbital-pretreated rats were examined 4 h after administration of each of these 13 agents for the presence of abnormal ("green") pigments. Using the extraction and purification methods described under "Experimental Procedures," characteristic abnormal pigments were found in rats treated with each of the 10 substrates bearing a terminal acetylenic function (Fig. 1). In contrast, no abnormal pigments were isolated by the standard procedure from rats treated with the three agents in which the acetylenic moiety was disubstituted, even though these agents caused *in vitro* loss of cytochrome P-450. In one of these three cases, that of 3-pentyn-2-ol, this failure was due to the fact that rats receiving the usual 200 mg/kg dose of the agent died shortly after drug administration. It may be that this exceptional toxicity is related to the unique presence in this substrate of an oxidizable hydroxyl function adjacent to the acetylenic group, a pairing of functionalities which may lead to formation of a reactive carbonyl-conjugated acetylene. Large scale incubation of norethisterone with a microsomal enzyme preparation resulted in isolation of an abnormal pigment identical in its chromatographic and spectroscopic properties with that obtained from *in vivo* administration of the drug.

Abnormal hepatic pigments were analyzed by thin layer and high pressure liquid chromatography. The free-base form of 3 of the 10 pigments was resolved into two components by both of these techniques. The free-bases of the other seven pigments chromatographed as single bands, although the presence of unresolved isomers within these bands is suggested by frequently observed asymmetry in the high pressure liquid chromatography peaks. High pressure liquid chromatographic analysis of the zinc complexes was also performed, although these were not resolved into more than one peak even in those instances where two components were known to be present from the free-base chromatographic data. The high pressure liquid chromatographic analysis of a mixture of metal-free and zinc-complexed forms of the 3-phenoxy-1-propyne pigment, a typical example, is given in Fig. 2. The three pigments which could be resolved into two components were those engendered by norethisterone (34), 1-ethynylcyclohexanol, and 1-ethynylcyclopentanol. These three substrates were the only ones with a tertiary hydroxyl group adjacent to a terminal ethynyl function. As already reported for norethisterone (34), the two components of each of the three pigments were not interconverted by complexation with zinc acetate and subsequent demetalation in 5% H_2SO_4/methanol. Only the single component used as a starting material was obtained after this reaction cycle. Nevertheless, even though not easily interconvertible, the evidence to be outlined below strongly suggests that the two components of each of these pigments are in fact closely related isomeric structures.

The electronic absorption spectrum of each of the purified pigments, and of each of the two components of a pigment when these were resolved, has been determined both in the

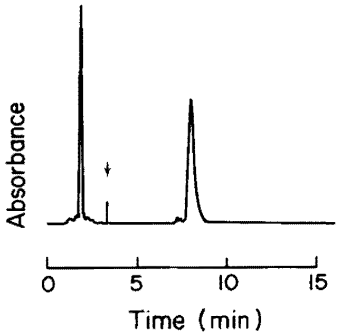


FIG. 2. **High pressure liquid chromatographic analysis of a mixture of the metal-free and zinc-complexed pigment isolated from rats treated with 3-phenoxy-1-propyne.** A mixture of the metal-free and zinc-complexed pigment, obtained by addition of zinc acetate to pigment isolated as described under "Experimental Procedures," was chromatographed on a Whatman Partisil 10-PAC column. The first solvent, hexane:tetrahydrofuran:methanol, 10:3:1, was replaced at the point shown by an *arrow* with methanol:tetrahydrofuran, 4:1. A 3 ml/min solvent flow rate was used. The detector was at 417 nm. The first peak is due to uncomplexed product and the second is due to the zinc complex.

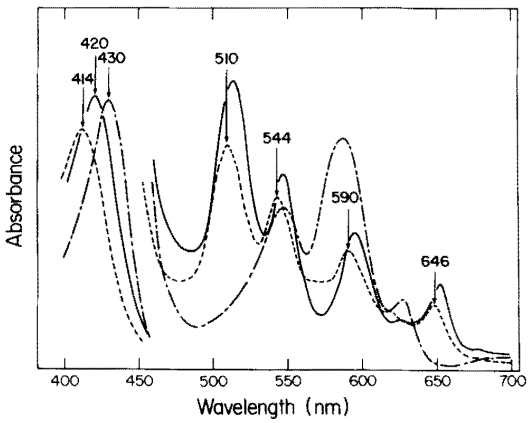


FIG. 3. **Electronic absorption spectra.** Electronic (ultraviolet-visible) absorption spectra in chloroform of (*a*) the free base of the pigment obtained with 3-phenyl-1-propyne (——); (*b*) the free base of the pigment obtained with acetylene gas (- - -); and (*c*) the zinc complex of the 3-phenyl-1-propyne pigment (·—·).

metal-free and zinc-complexed state. Within experimental tolerance (approximately ± 2 nm), the spectra of all of the uncomplexed pigments were essentially identical with two exceptions. The free-base of the pigment obtained with acetylene gas exhibited a Soret band at 414 nm rather than at 420 nm as in the spectra of the other pigments. This can be seen by comparing (Fig. 3) the spectrum of the 3-phenyl-1-propyne pigment, a typical case, with that of the acetylene pigment. The second exception is provided by the phenylacetylene pigment, for which a reproducible free-base spectrum has not been obtained due to chemical instability of the chromophore. The spectra of the zinc complexes of the pigments were also essentially identical, in this case with no exceptions (Fig. 3). The zinc complex of the pigment obtained with phenylacetylene, in contrast to the free-base form, exhibited a stable spectrum which could not be differentiated from those of the other zinc-complexed pigments. Correlation of the spectra of both the metal-free and zinc-complexed forms of the pigments with the corresponding spectra of authentic porphyrins (42) leaves no doubt that the basic chromophore in the pigments is that of a porphyrin ring.

Field desorption mass spectra were obtained for the free base of eight of the isolated pigments, including separate spectra for each of the two resolved components in the pigments obtained with 1-ethynylcyclohexanol and 1-ethynylcyclopentanol. A mass spectrum was also obtained of the zinc complex of each of the pigments (Table I). The mass spectrum of the pigment produced by treatment with acetylene gas is reproduced in Fig. 4. In general, these spectra are characterized by the presence of a variable ratio molecular ion doublet and by a minimal amount of peaks due to molecular fragmentation. The molecular ion doublet is due to desorption of protonated and unprotonated molecular species, the monoprotonated form being favored by higher emitter currents in the mass spectrometer. Protonated and alkali metal-complexed molecular ions are frequently observed in field desorption mass spectrometry (43). A sodium-complexed molecular ion was in fact observed for the norethisterone pigment (34). An instructive exception to the observation of a molecular ion doublet was provided by the 3-(2,4-dichlorophenoxy)-1-propyne pigment, which exhibited the molecular ion cluster ex-

TABLE I

Analysis of isolated hepatic pigments by field desorption mass spectrometry

The observed molecular ion for each purified pigment, after subtraction of the mass unit(s) due to the complexed proton or sodium cation (shown in parentheses), is given in the table. These data are given both for the free-base and for the zinc complex obtained on addition of zinc acetate. The principal peak in the zinc complex molecular ion cluster, corresponding to complexation with the major isotope of zinc (^{64}Zn), is listed.

Substrate (M_r)	Predicted molecular ion (m/e)	Observed molecular ion (m/e)	
		Free base	^{64}Zn complex
A (124)	730	730 (H^+)	792 (H^+)
B (110)	716	716 (H^+)	778 (H^+)
D (298)	904	886 (Na^+)	
F (200)	806	806 (H^+)	868 (H^+)
H (132)	738	738 (H^+)	800 (H^+)
I (130)	736	736 (H^+)	798 (H^+)
J (116)	722	722 (H^+)	784 (H^+)
M (26)	632	632 (H^+)	694 (H^+)

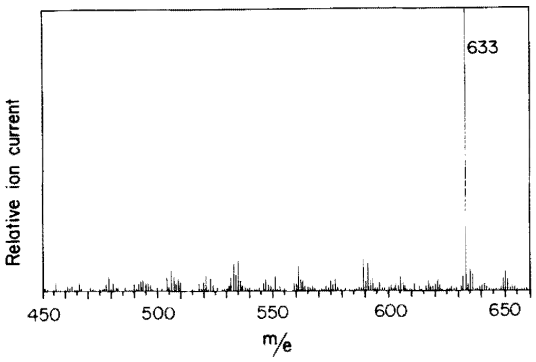


FIG. 4. **Field desorption mass spectrum of the hepatic pigment isolated from phenobarbital-pretreated rats exposed to acetylene gas.** Administration of the agent and isolation of the pigment are described under "Experimental Procedures." The principal peak observed in the spectrum (m/e 633) corresponds to monoprotonated molecular ion.

pected of a mixture of protonated and unprotonated ions of a substance containing 2 chlorine atoms (44). The natural abundance ratio of isotopes (45) was also reflected in the molecular ion patterns of the zinc complexes. Again, the zinc complex of the 3-(2,4-dichlorophenoxy)-1-propyne pigment gave an unusually complex molecular ion pattern, as expected for a structure containing 1 zinc and 2 chlorine atoms. The molecular ion values tabulated in Table I correspond to the unprotonated molecular species. The values given for the zinc complexes and for the chlorine-containing compound are those of the major peak in the molecular ion clusters and, thus, correspond to the presence of ^{64}Zn and ^{35}Cl. In those cases where a pigment was resolved into two components, both components gave the same mass spectrometric data so that the value in Table I is valid for both components.

DISCUSSION

The importance of the acetylenic group in norethisterone and ethynylestradiol for the destructive interaction of these substrates with hepatic cytochrome P-450 was first noted by White and Müller-Eberhard (26), who reported that destructive activity was lost on replacement of the ethynyl function with a hydrogen atom or an ethyl group. Our subsequent demonstration that the ethynyl function could also not be replaced by a vinyl moiety confirmed the specific role of the carbon-carbon triple bond in the destructive action of these hormonal steroids (34). The observation that nonsteroidal acetylenes also mediated the loss of cytochrome P-450 furthermore demonstrated that the sterol framework was not an essential component of the destructive interaction (34, 41). The more extensive structure-activity study described here unequivocally establishes that the potential to interact destructively with cytochrome P-450 is an intrinsic property of the acetylenic group itself and that although the interaction can be attenuated or suppressed by the framework into which the triple bond is incorporated, no other substrate feature is essential for cytochrome P-450 destruction. Thus, this monooxygenase system is efficiently destroyed by monosubstituted acetylenes in which the attached carbon is not only mono- and di-, but also trisubstituted, a result which excludes destructive mechanisms based on formation of a delocalized radical by abstraction of an allylic hydrogen atom. The destructive activity of compounds such as 1-ethynylcyclohexane and phenylacetylene clearly shows that a vicinal hydroxyl function is also not required, although involvement of the hydroxyl group in oxidative metabolism of the triple bond in ethynyl sterols (46, 47) leaves open the possibility that it may intervene when present (34). Nevertheless, the lack of structural specificity implicit in the observation that all of the substrates listed in Fig. 1 destroyed cytochrome P-450 convincingly argues that the nature of the structure bearing the acetylenic moiety is not a primary determinant of destructive activity, although the decreased effectiveness of phenylacetylene and *N*-(1,1-dimethyl-2-propynyl)-3,5-dichlorobenzamide does show that the surrounding structure can interfere with the destructive process. The intrinsic activity of the acetylenic moiety is perhaps most strikingly demonstrated by the destructive action of acetylene itself (41), whereas the loss of cytochrome P-450 caused by the disubstituted acetylenes 3-pentyn-2-ol, 4-methyl-2-octyn-4-ol, and 2-hexyne shows that it is the unsaturated bonds of the acetylenic group and not the acidic acetylenic proton which are responsible for the destructive interaction with cytochrome P-450, since in these substrates the acidic acetylenic proton is absent.

The destruction of cytochrome P-450 by 2-isopropyl-4-pentenamide, ethynyl sterols, and acetylene gas requires NADPH, oxygen, and catalytically competent enzyme (26, 41, 48). However, the most characteristic feature of the inactivation of cytochrome P-450 by these particular agents, the feature which uniquely distinguishes their mechanism, is the accompanying loss of the enzyme prosthetic heme moiety and the accumulation of abnormal hepatic pigments derived from that moiety (26, 34, 41, 48). The destruction of cytochrome P-450 by monosubstituted acetylenes has been clearly shown by this study to be mediated by this mechanism, both by demonstration that in every instance NADPH is required and, more definitively, that all of the new monosubstituted substrates examined *in vivo* yielded hepatic pigments indistinguishable by absorption spectroscopy (Fig. 3) from that previously obtained with norethisterone (34). This identity in electronic absorption spectra, true both for the free-base and zinc-complexed forms of the pigments, was also valid for the two resolved components of the pigments obtained with norethisterone, 1-ethynylcyclohexanol, and 1-ethynylcyclopentanol.

The *in vitro* destruction of cytochrome P-450 was mediated equally well by monosubstituted and disubstituted acetylenes (Fig. 1). In contrast, however, abnormal hepatic pigments were only observed in rats which had been treated *in vivo* with monosubstituted acetylenes or with acetylene gas. Analogous hepatic pigments were not found in rats which had been treated with disubstituted acetylenes. This difference in the action of mono- and disubstituted acetylenes is an important one since pigment formation is intimately associated with the mechanism which governs destruction of cytochrome P-450 by acetylene gas and monosubstituted acetylenes. In the absence of such pigments, the mechanism by which disubstituted acetylenes destroy the enzyme can not be unambiguously linked to the mechanism defined here (see below) for the action of terminal acetylenes.

In preliminary studies using radiolabeled norethisterone, we have demonstrated that the resulting abnormal pigment is a covalent adduct of the drug with the porphyrin chromophore derived from heme (34). However, little structural information beyond the presence of a covalent link could be inferred from the demonstration that radioactivity remained associated with the chromophore throughout a series of chromatographic procedures. Although a field desorption mass spectrum of the norethisterone adduct was obtained, a clear rationale for the observed molecular ion could not be formulated (34). The limitations of this earlier study have now been transcended by mass spectrometric examination of seven other pigments obtained in this investigation including, where appropriate, separate mass spectra for chromatographically resolved components. The resulting data (Table I) unequivocally confirm that all of these agents act by a common mechanism in which 1:1 covalent alkylation by the causative agent of the protoporphyrin IX skeleton of prosthetic heme leads to inactivation of the enzyme and formation of the abnormal hepatic pigment. In each case, except that of norethisterone itself, the molecular ion observed corresponds exactly to that expected for the sum of the molecular weights of the dimethyl ester of protoporphyrin IX (M_r = 590) plus the acetylenic agent plus 16 mass units, these last almost certainly due to an oxygen atom. The dimethyl ester of the porphyrin, rather than the free acid, is expected in this summation since the carboxyl groups are methylated during treatment with H_2SO_4/methanol. Formation of the dimethyl ester has been specifically confirmed by deuterium-labeling studies in the case of the porphyrin adduct with 2-isopropyl-4-pentenamide (33). The iron atom is also lost from the adduct during the acidic extraction procedure. A similar sum of structural fragments is required by the

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.