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Destruction of Cytochrome *P*-450 by Ethylene and Other Olefins

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SUMMARY

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Hepatic microsomal cytochrome *P*-450 from phenobarbital-pretreated rats is destroyed *in vitro*, in the presence of NADPH, by terminal (monosubstituted) olefins such as 4-ethyl-1-hexene and 1-heptene. The destructive interaction apparently depends on no other factor than the presence of a carbon–carbon double bond, since ethylene gas is an effective destructive agent. Hepatic "green" pigments are formed on the administration of terminal olefins to phenobarbital-pretreated rats. These pigments are essentially identical, by electronic absorption spectroscopy, to that previously reported to be formed on the administration of 2-isopropyl-4-pentenamide. Although *cis*- and *trans*-2-nonene exhibit marginal destructive activity, no abnormal pigments were found after *in vivo* administration. No cytochrome *P*-450 loss is observed after incubation with styrene, cyclohexene, 2-methyl-1-heptene, and 3-hexene, a result which suggests that steric and electronic factors can suppress the destructive interaction. The epoxides of three of the terminal olefin substrates have been synthesized and shown not to intervene in destruction of the enzyme by the parent olefins. Mechanistic and physiological implications of these observations are discussed.

INTRODUCTION

Phenobarbital-inducible cytochrome *P*-450 monooxygenases are selectively, perhaps specifically, destroyed during the attempted metabolism of 2-isopropyl-4-pentenamide (Allylisopropylacetamide) (1, 2), 2-phenyl-4-pentenamide (3), 2,2-diethyl-4-pentenamide (4), secobarbital[2] (5), and allobarbital[2] (3, 5). A γ-unsaturated amide substructure, the only common feature in these substrates, is likely to be involved in the destructive interaction. Support for this contention is provided by loss of destructive activity on saturation of the homoallylic double bond (3, 5). The distinguishing feature of the mechanism by which these substrates destroy cytochrome *P*-450, a suicidal mechanism which requires catalytically competent enzyme (6), NADPH (5), and oxygen,[3] is the accompanying formation of abnormal ("green") hepatic pigments (1, 2, 7). These pigments, identified as porphyrins by their characteristic electronic absorption spectra (8, 9), are derived from the prosthetic heme moiety of the inactivated enzyme (7). Recent studies in this laboratory have conclusively established that the pigment obtained with 2-isopropyl-4-pentenamide is a 1:1 covalent adduct of this substrate with protoporphyrin IX (10–12). Cytochrome *P*-450 is therefore destroyed through alkylation of its prosthetic heme by a reactive species generated *in situ*.

In order to define the features of the γ-unsaturated amide substructure essential for cytochrome *P*-450 destruction, we have examined the effect on destructive activity of altering the amide group. The finding that the *N*-phenyl and *N,N*-dimethyl derivatives of 2-isopropyl-4-pentenamide remained active[3] led us to replace the amide with a methyl ester function (12). This methyl ester not only destroyed cytochrome *P*-450, but also gave an adduct with prosthetic heme which, after isolation, was indistinguishable from that obtained with the amide by three criteria: chromatographic properties, electronic absorption spectrum, and mass-spectrometrically determined molecular weight. The apparent identity of these adducts, and particularly the molecular ion equivalence, suggested the possible participation of the substrate carbonyl groups in the destructive event (12). The observation that the amide in 2-isopropyl-4-pentenamide could be replaced by a methyl ketone without loss of activity was consistent with this suggestion, although the finding that the carbonyl could be replaced by a methyl ether

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[2] Abbreviations used: secobarbital, 5-allyl-5-isobutylbarbituric acid; allobarbital, 5,5-diallylbarbituric acid; ir, infrared; NMR, nuclear magnetic resonance; CIMS, chemical ionization mass spectrum.

[3] P. R. Ortiz de Montellano and B. A. Mico, submitted.

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was not (11). However, since it was not determined whether abnormal green pigments were formed with the ketone and the ether, it was not possible to exclude the intervention of a different mechanism. We now report not only the isolation of such pigments with these two substrates, but also the more significant discovery that analogous pigments accompany destruction of the enzyme by substrates bearing no functionality other than a carbon–carbon double bond. Indeed, we have found that ethylene, the most elementary of olefins, efficiently mediates the destruction of cytochrome *P*-450.

MATERIALS AND METHODS

Substrates. A sample of 2-isopropyl-4-pentenamide was kindly provided by Hoffman–LaRoche, Nutley, N.J. Chemical Samples Co., Columbus, Ohio, was the source of 4-ethyl-1-hexene, 3-ethylhexane, 3-methyl-1-octene, 2-methyl-1-heptene, 3,3-dimethyl-1-hexene, *trans*-2-nonene, *cis*-2-nonene, *trans*-3-hexene, and *cis*-3-hexene; 1-heptene was provided by Aldrich Chemical Co., Milwaukee, Wis.; heptane and cyclohexene were from Eastman Organic Chemicals, Rochester, N.Y.; ethylene and ethane (C.P. grade, 99.5% pure) were purchased from Matheson Gas Products, Newark, Calif. All liquid samples were distilled prior to use.

Acetylene-free ethylene was generated by a procedure based on the classic one of Erlenmeyer and Bunte (13). A 6:1 (v/v) mixture of concentrated H_2SO_4 and freshly distilled anhydrous ethanol (175 ml total) was placed in a 1-liter round-bottom flask equipped with an addition funnel containing 2:1 (v/v) H_2SO_4–ethanol. The flask was also fitted with a dry ice–acetone condenser, the top of which was connected to a bubbler inserted into a flask containing 0.1 N phosphate buffer (pH 7.4). The system was so arranged that ethylene gas generated in the round-bottom flask was stripped of entrained vapors by the dry-ice condenser and the buffer wash before being passed into incubation mixtures. The hydrocarbon gas was generated by heating the round-bottom flask in an oil bath to 160–170°C and then, with vigorous magnetic stirring, slowly adding the mixture from the addition funnel dropwise. *Caution*: Care must be taken not to add the mixture too rapidly since this may result in rupture of the flask due to a surge in gas formation. The gas evolved rapidly decolorizes dilute (0.5%) potassium permanganate solutions.

Ethylene-free ethane was generated by the dropwise addition of ethyl magnesium bromide (3 M in diethyl ether, obtained from Ventron Corp., Danvers, Mass.) to anhydrous ethanol in a vigorously stirred two-neck round-bottom flask. The evolved gas was passed through a dry-ice condenser and a buffer wash, as described previously for ethylene, before being gently bubbled into incubation mixtures.

3-Isopropyl-5-hexen-2-one. Methyl lithium in diethyl ether (2.3 M, 62 mmol) was slowly added by syringe to a stirred solution of 2-isopropyl-4-pentenoic acid (4.0 g, 28.2 mmol) in 100 ml dry THF at −78°C under nitrogen (see Ref. 14). After stirring for 25 h at 25°C, water (100 ml) and diethyl ether (300 ml) were cautiously added. The organic layer, washed with 10% NaOH and dried over anhydrous sodium sulfate, was concentrated under vacuum. The resulting yellow oil was purified by low-pressure chromatography on a Merck silica gel 60 column, using 5% ethyl acetate in hexane as solvent (8 ml/min flow rate). Pure ketone (2.10 g, 53%) was thus obtained: ir (film) 1725 cm^{-1} (C=O); NMR (in $CDCl_3$, Me_4Si standard) δ 0.90 (*d*, 6H, J = 6 Hz, iPr-methyls), 1.07–2.50 (*m*, 4H, internal CH), 2.10 (*s*, 3H, $COCH_3$), 4.93–5.25 (*m*, 2H, $C{=}CH_2$), and 5.33–6.00 ppm (*m*, 1H, —CH=C); CIMS *m/e* 141 $(M+1)^+$.

Methyl 2-isopropyl-4-pentenyl ether. Reduction of 2-isopropyl-4-pentenoic acid with $LiAlH_4$ gave 2-isopropyl-4-penten-1-ol. A solution of the alcohol (1.50 g, 10.6 mmol) in 30 ml of anhydrous diethyl ether was cautiously added at 0°C under nitrogen to a stirred suspension of hexane-washed sodium hydride (1.5 equivalents) in the same solvent. Methyl iodide (3.0 g, 21.1 mmol) was then added and the reaction was stirred at 25°C for 42 h before water was cautiously added. The organic layer, after washing with water, drying over magnesium sulfate, and solvent removal, yielded 1.05 g of oil which was purified by chromatography on 10% water-deactivated silica gel (5% ethyl acetate–hexane solvent). The ether was obtained in 56% yield: ir (film) no carbonyl or hydroxyl; NMR (as previously) δ 0.9 (*d*, 6H, J = 6.5 Hz, iPr-methyls), 1.07–2.47 (*m*, 4H, internal CH), 3.32 (*s*, 3H, OCH_3), 3.35 (*d*, 2H, J = 2 Hz, CH_2O), 4.78–5.33 (*m*, 2H, $C{=}CH_2$), and 5.37–6.17 ppm (*m*, 1H, —CH=C); CIMS *m/e* 143 $(M+1)^+$. *Anal.* Calcd. for $C_9H_{18}O$: C, 76.06; H, 12.68. Found: C, 76.35; H, 12.68.

4-Ethyl-1,2-epoxyhexane. To a solution of *m*-chloroperbenzoic acid (6.16 g, 54 mmol) in 100 ml CH_2Cl_2 was added 4.0 g (38 mmol) of 4-ethyl-1-hexene at 25°C. After stirring overnight, excess peracid was destroyed by vigorously stirring the organic solution with 10% aqueous $NaHSO_3$ for 1 h. The organic layer, washed with saturated $NaHCO_3$, dried over $MgSO_4$, and stripped of solvent, gave a yellow oil which was distilled (55°C, 20 Torr) to give 2.28 g (47%) of clear, colorless epoxide: ir (film) 1238, 863 cm^{-1}; NMR (as previously) δ 0.88 (*t*, 6H, J = 7.5 Hz, CH_3's), 1.05–1.60 (*m*, 7H, internal CH), 2.44 (*q*, 1H, J = 3 Hz, epoxy CH), and 2.55–3.00 ppm (*m*, 2H, epoxy CH_2); CIMS *m/e* 129 $(M+1)^+$.

1,2-Epoxyheptane. This epoxide, prepared as previously, was identical to that previously reported by this laboratory (15).

In vitro cytochrome P-450 destruction. The loss of spectroscopically observable cytochrome *P*-450 on incubation of hepatic microsomes from phenobarbital-induced rats with test substrates was determined as previously described (6, 10, 11). Incubations contained the following: microsomal protein (1 mg/ml), KCl (150 mM), EDTA (1.5 mM), and NADPH (1.0 mM) in 0.1 N Na/K phosphate buffer (pH 7.4). Protein concentrations were measured by the Lowry procedure (16). Substrates were added at a nominal 10 mM concentration except for ethylene and ethane, which were gently bubbled through the microsomal suspensions for 1–10 min (see text). The gases were introduced *via* a syringe needle inserted through a septum covering the incubation flask, a second needle being inserted to allow pressure equalization. Both

needles were withdrawn before the addition by syringe of NADPH, the reagent initiating the incubation. Sufficient oxygen remained in the sealed flask for the enzymatic reaction to occur. Only minor NADPH-dependent cytochrome *P*-450 losses were observed in the absence of substrates (see Table 4), indicating that lipid peroxidation was effectively suppressed by the added EDTA. A lipid peroxidation control was performed with each set of incubations. The values in the tables have been corrected for lipid peroxidation by subtraction of these small-to-negligible substrate-independent losses. Destruction of cytochrome *P*-450 by each substrate in the absence of NADPH was also measured to control for destructive processes which did not require catalytic participation of the enzyme. Formation of cytochrome *P*-420 as a result of cytochrome *P*-450 loss was shown to be negligible with several of the substrates (28).

Isolation and spectroscopic characterization of porphyrin-substrate adducts (hepatic pigments). The procedure developed for isolation of norethisterone-derived pigments (17) was adapted for this study since it is simpler in procedural terms than that used in our previous studies with 2-isopropyl-4-pentenamide (12).

Sprague–Dawley male rats (three to five rats per substrate) weighing approximately 250 g were injected intraperitoneally for 4 days with a daily phenobarbital dose of 80 mg/kg. Test substrates were injected intraperitoneally on the fifth day, a 400 μl/kg dose of the oils being used. Ethylene gas was administered by placing the rats in a chamber through which was passed a 1:1 (v/v) mixture of ethylene and air at a flow rate of approximately 2 liters/min for 3 h. Four hours after the injection of oils, or at the end of ethylene gas administration, rats were decapitated and their livers were perfused with ice-cold isotonic saline solution. The excised livers, homogenized in 5% H_2SO_4/methanol (10 ml/g liver), were allowed to stand overnight in the dark at 4°C. The resulting mixture was filtered and equivalent volumes of water and dichloromethane were added. The organic layer was separated, washed with water and saturated bicarbonate solution, and dried (Na_2SO_4). Solvent removal on a rotary evaporator gave a residue to which was added a 0.5% solution of zinc chloride in 5% methanol/chloroform. The resulting zinc-complexed pigment, after solvent removal, was chromatographed on a silica gel G preparative plate (Analtech), using 3:1 chloroform/acetone as solvent. The absorption spectrum of the green (red-fluorescing) band, after extraction with acetone and solvent removal, was recorded in chloroform solution on a Varian Cary 118 spectrophotometer. The metal-free porphyrin was then obtained by stirring the zinc complex in 5% H_2SO_4/methanol for 15 min. The addition of equal volumes of methylene chloride and water, separation and aqueous washing of the organic layer, drying over anhydrous sodium sulfate, and solvent removal gave the desired free base.

In vitro pigment formation with acetylene-free ethylene. The livers from four phenobarbital-pretreated rats were homogenized in 0.1 N phosphate buffer (pH 7.4), using a volume of buffer (in milliliters) equal to the liver weight (in grams). The 9000*g* supernatant obtained from this homogenate was directly used for the incubation. The final incubation mixture also contained $MgCl_2$ (3 mM), glucose-6-phosphate (3 mM), and NADPH (2 mM). Ethylene gas, generated from ethanol as already described, was gently bubbled through the mixture for 10 min before the flask was sealed and the mixture was incubated at 37°C for 30 min. The incubation mixture was then added to 5% H_2SO_4/methanol (10 ml/g of original liver) and the pigment was isolated as described in the preceding section. The absorption spectra of the pigment thus obtained, as both the free base and the zinc complex, corresponded to the spectra of the pigment isolated after the *in vivo* administration of commercial ethylene.

Gas-chromatographic epoxide analysis. The epoxide was partitioned into a volume of ether equal to the volume of the incubation mixture or, in control experiments, of a standard solution of the epoxide in 0.1 N phosphate buffer (pH 7.4). Aliquots of the ether extracts were analyzed by gas chromatography on a Varian 2100 instrument equipped with a flame-ionization detector and a 6-ft 3% OV-225 column. At a carrier gas flow rate of 16 ml/min and a temperature of 100°C, 4-ethyl-1,2-epoxyhexane had a retention time of 56 s. At 70°C, 1,2-epoxyheptane had a retention time of 100 s. The concentration in the incubation mixture was determined by peak-height comparison of its ether extract with that of the extract from a standard solution. No correction has been made for nonvalent binding of epoxide to microsomes.

RESULTS

Destruction of cytochrome P-450 by terminal monosubstituted double bonds. The methyl ketone and methyl ether analogues of 2-isopropyl-4-pentenamide, 2-isopropyl-5-hexen-2-one and methyl 2-isopropyl-4-pentenyl ether, destroy cytochrome *P*-450 as effectively as the parent amide (Table 1). The enzyme was also destroyed by 4-ethyl-1-hexene, 1-heptene, and 3-methyl-1-octene, three of the five terminal (monosubstituted) olefins investigated, although no detectable enzyme loss was observed with 3,3-dimethyl-1-hexene and styrene, the other two olefins of this type. Control experiments established that cytochrome *P*-450 was not being converted to cytochrome *P*-420. In the absence of NADPH, erratic but occasionally substantial (up to 30%) losses of the enzyme were promoted by 4-ethyl-1-hexene, its saturated analogue 3-ethylhexane, and 1-heptene (see Table 4), but not (<5%) by any other hydrocarbon in this study. The values in Table 1, however, are the spectrophotometrically determined difference in enzyme loss between incubations with and without NADPH, and are thus a measure of NADPH-dependent destruction. Although the precise nature of the NADPH-independent effect with the three exceptional substances has not been defined, the fact that reduction of the concentration of 4-ethyl-1-hexene and 1-heptene from 10 to 1 mM obliterates the NADPH-independent effect (but has little effect on NADPH-dependent destruction; Table 4) suggests that it may result from perturbation of the membrane. The NADPH-dependent and -independent effects are in any case clearly differentiated by this concentration dependence and by the activity of the saturated hydrocarbon (see the following).

TABLE 1

In vitro destruction of hepatic microsomal cytochrome P-450 by experimental substrates

The procedure is given in the experimental section. The values indicate percentage loss of cytochrome *P*-450 due to incubation of substrates for three different time periods with hepatic microsomes from phenobarbital-pretreated rats. The values have been corrected for minor (1–3%) losses due to lipid peroxidation and, in the case of substrates 5, 7, and 8, for significant NADPH-independent losses. Each value is the average of a minimum of three determinations and is given with its standard deviation. Values of less than 5% are not considered experimentally significant. The structures of the substrates are given in Fig. 1. The last column indicates whether an abnormal hepatic pigment was found (yes) or not (no) or whether the experiment was not performed (—).

Substrate (10 mM)	Percentage cytochrome *P*-450 loss			*In vivo* pigment formation
	10 min	20 min	30 min	
2-Isopropyl-4-pentenamide (1) (12)	22 ± 5	28 ± 7	36 ± 8	Yes
Methyl 2-isopropyl-4-pentenoate (2) (12)	30 ± 2	37 ± 8	38 ± 4	Yes
3-Isopropyl-5-hexen-2-one (3)	27 ± 1	32 ± 4	38 ± 3	Yes
Methyl 2-isopropyl-4-pentenyl ether (4)	25 ± 5	34 ± 4	38 ± 4	Yes
4-Ethyl-1-hexene (5)	20 ± 3	31 ± 6	38 ± 6	Yes
3-Ethylhexane (6)	1 ± 1	1 ± 1	1 ± 1	—
1-Heptene (7)	17 ± 7	17 ± 7	17 ± 6	Yes
Heptane (8)	1 ± 1	1 ± 2	1 ± 2	—
3-Methyl-1-octene (9)	14 ± 1	20 ± 3	21 ± 3	Yes
Cis 2-nonene (10)	6 ± 2	8 ± 2	10 ± 3	No
Trans 2-nonene (11)	7 ± 3	8 ± 3	10 ± 3	No
3,3-Dimethyl-1-hexene (12)	1 ± 1	1 ± 2	1 ± 2	—
Styrene (13)	0	0	0	No
2-Methyl-1-heptene (14)	1 ± 1	1 ± 1	1 ± 1	—
Cyclohexene (15)	1 ± 2	3 ± 2	4 ± 3	No
Cis 3-hexene (16)	3 ± 0	4 ± 1	5 ± 1	—
Trans 3-hexene (17)	3 ± 1	4 ± 1	4 ± 1	—

Abnormal hepatic pigments accumulate in phenobarbital-pretreated rats after the administration of 2-isopropyl-5-hexene-2-one, methyl 2-isopropyl-4-pentenyl ether, or any of the three terminal olefins which destroy cytochrome *P*-450 *in vitro* (Table 1). In contrast, no such pigment was found after the administration of styrene, a terminal olefin which is inactive *in vitro*. The presence of abnormal pigments was generally signaled by a subtle discoloration, after removal and perfusion, of the livers of treated rats. The pigments were extracted with acidic methanol, a process which results in methylation of porphyrin carboxyl groups and extrusion of porphyrin metal ligands (12). The electronic (ultraviolet-visible) absorption spectra of the purified pigments, in both the metal-free and the zinc-complexed form, are given in Table 2. The absorption spectra of the zinc complexes of the pigments obtained with the ketone and ether analogues of 2-isopropyl-4-pentenamide are essentially identical to that of the zinc complex of the pigment obtained with the amide itself. Furthermore, this analogy is also valid for the zinc complexes of the pigments due to 4-ethyl-1-hexene, 3-methyl-1-octene, and 1-heptene. The metal-free spectra of all of these pigments are also essentially identical to each other (Table 2).

TABLE 2

Absorption maxima of the metal-free and zinc-complexed pigments isolated from rats treated with unsaturated substrates

The peaks in spectra of the metal-free pigments exhibited a progressive decrease in intensity going from lower to higher wavelengths, the resulting etio intensity pattern being essentially identical to that previously published (10–12). The peak ratios in the spectra of the zinc complexes were also approximately the same as those reported for the zinc complex of the 2-isopropyl-4-pentenamide pigment (10, 11).

Substrate	Free-base maxima	Zinc-complex maxima
	nm	*nm*
2-Isopropyl-4-pentenamide	417,512,545,594,652	431,547,591,634
Methyl 2-isopropyl-4-pentenoate	417,512,545,594,652	431,547,591,630
3-Isopropyl-5-hexen-2-one	418,512,546,592,649	434,547,591,633
Methyl 2-isopropyl-4-pentenyl ether	416,513,547,591,650	434,548,590,633
4-Ethyl-1-hexene	419,510,546,594,650	432,546,591,633
3-Methyl-1-octene	418,511,546,594,647	431,546,590,630
1-Heptene	418,510,545,593,648	432,546,587,630
Ethylene	415,513,545,591,652	432,546,591,630

No NADPH-dependent loss of cytochrome *P*-450 was observed on incubation of heptane and 3-ethylhexane with hepatic microsomes (Table 1) although, as mentioned, some NADPH-independent losses were caused by 3-ethylhexane. The olefinic bond is thus implicated as a participant in the NADPH-dependent destructive interaction of the olefins with the enzyme.

Destruction of cytochrome P-450 by ethylene gas. The destruction of cytochrome *P*-450 by olefins, reported previously, led us to investigate the effect of ethylene. Initial experiments were carried out with high-grade commercial ethylene gas. As reported in Table 3, incubation of hepatic microsomes with ethylene in the presence of NADPH resulted in rapid destruction of cytochrome *P*-450, whereas no enzyme loss was observed in the absence of NADPH in either a differential or an absolute sense. One ambiguity, however, obscured the

TABLE 3

Destruction of cytochrome P-450 by ethylene gas

Ethylene and ethane, generated as described in the experimental section, were bubbled into the incubation mixtures at approximately the same rate for the time indicated before incubations were begun. Other details are given in Table 1. No loss of cytochrome *P*-450 was observed if NADPH was withheld from the incubation mixture. The data for generated ethylene are from one series of incubations.

Substance	Percentage cytochrome *P*-450 loss		
	10 min	20 min	30 min
Ethylene, commercial gas	26 ± 5	31 ± 2	31 ± 3
Ethylene, generated			
1 min	6	13	13
3 min	16	18	21
10 min	22	29	34
Ethane, generated (10 min)	1	1	4

clarity of this result. Commercial ethylene gas, even that of the highest grade, is reported by the manufacturer to contain a trace (approximately 10 ppm) of acetylene. Recent work by White (19), confirmed and extended in this laboratory,[4] has established that acetylene destroys cytochrome *P*-450. The possibility thus existed, even if unlikely due to the low concentration of the contaminant, that acetylene was exclusively responsible for the observed enzyme loss. In order to circumvent this ambiguity, pure ethylene was generated in the laboratory by dehydration of ethanol with concentrated sulfuric acid. The gas obtained, after passage through a dry-ice condenser and a buffer solution, was bubbled into incubation mixtures for 1, 3, and 10 min. A rough increase in the ethylene concentration in the incubations was thus achieved. As shown in Table 3, pure ethylene gas caused a concentration-dependent loss of cytochrome *P*-450. Experiments were also carried out with ethane gas to determine if the double bond was critical to the destructive process. Unfortunately, commercial ethane gas, although free of acetylene, does contain significant amounts of ethylene (approximately 0.03 mol% in the sample used). Not surprisingly, incubations with this gas led to substantial cytochrome *P*-450 destruction (not shown). This result, consistent with the conclusion that ethylene itself (rather than acetylene) was the active agent in the previous experiments, unfortunately does not resolve the question of double-bond involvement. Pure ethane was therefore generated in the laboratory by the reaction of ethyl magnesium bromide with ethanol. This saturated hydrocarbon was found to be inactive (Table 3), confirming that the unsaturated bond is required for enzyme destruction.

Commercial ethylene was administered to phenobarbital-pretreated rats by inhalation. A pigment was isolated from the livers of these animals whose zinc complex exhibited the same absorption spectrum as the previously discussed complexes. The spectrum of the metal-free pigment was also similar to that of the other pigments except for a slightly shifted Soret band (Table 2). Due to the large volumes of gas required for *in vivo* inhalation studies, this *in vivo* experiment could be carried out only with commercial ethylene gas. To confirm that *in vivo* pigment formation primarily reflected the activity of ethylene and not that of the trace of acetylene in the commercial gas, the formation of pigment *in vitro* using acetylene-free ethylene was investigated. Large-scale incubation of chemically generated ethylene with rat liver 9000*g* supernatant in effect gave a pigment indistinguishable from that obtained after *in vivo* gas administration, clearly demonstrating that ethylene itself mediated pigment formation.

Destruction of cytochrome P-450 by compounds with disubstituted double bonds. Six hydrocarbons incorporating a disubstituted double bond have been examined in this study, two of which proved totally inactive at the usual 10 mM concentration. No *in vitro* cytochrome *P*-450 loss was observed with 2-methyl-1-heptene, in which both substituents are on the same end of the double bond, or with cyclohexene, in which the double bond is part of a ring system (Table 1). Small but detectable decreases in cytochrome *P*-450 content were produced, in the presence of NADPH, by *cis*-2-nonene and *trans*-2-nonene. Analogous incubations with *cis*-3-hexene and *trans*-3-hexene, however, in which the double bond is two rather than one atom removed from the end of the hydrocarbon chain, yielded only experimentally insignificant cytochrome *P*-450 losses. Abnormal hepatic pigments were not found by the usual procedure after the administration of cyclohexene, *cis*-2-nonene, or *trans*-2-nonene to phenobarbital-pretreated rats. It is not possible to say, on the basis of the available evidence, whether the failure to find pigments with the two nonenes is due to their low destructive activity or to the fact that no pigments are formed by their destructive action.

Assay for destruction of cytochrome P-450 by epoxides. The possible epoxide metabolites of 4-ethyl-1-hexene and 1-heptene, 4-ethyl-1,2-epoxyhexane and 1,2-epoxyheptane, were chemically synthesized and their interaction with hepatic cytochrome *P*-450 was investigated. The change in cytochrome *P*-450 content of hepatic microsomes after incubation with each of these two epoxides is summarized in Table 4. Marginal but significant losses of cytochrome *P*-450 occurred, in the presence of NADPH, when the epoxides were present at 10 mM concentrations. However, this destruction became insignificant in the case of 4-ethyl-1,2-epoxyhexane when the concentration was reduced to 1 mM, a concentration at which the activity of the parent olefin was only slightly diminished (Table 4). With this same substrate, even at the higher concentration, there was little NADPH-independent loss of cytochrome *P*-450. On the other hand, the activity of 1,2-epoxyheptane was essentially the same in the presence or absence of NADPH. In neither case, however, was the marginal activity of the epoxide sufficient to account for the much greater destruction of the enzyme mediated by the corresponding parent olefin.

To strengthen these results, the incubations with 10 mM concentrations of the two epoxides used to obtain the data in Table 4 were extracted at the end of the experiment with diethyl ether. Gas-chromatographic analyses of these extracts, corrected for the volume of the incubation mixture utilized in cytochrome *P*-450 determinations, established that a minimum of 42% of the epoxide initially present survived the incubation in the case of 4-ethyl-1,2-epoxyhexane. The corresponding figure for 1,2-epoxyheptane was 37%. The concentration of these epoxides after 45 min of incubation was therefore no less than approximately 4 mM, a concentration at which the olefins still exhibit essentially undiminished activity.

One further series of experiments was performed to establish that added epoxides were able to reach the active site of the enzyme. In addition to the two epoxides discussed previously, the epoxide derived from methyl 2-isopropyl-4-pentenoate (12) was also examined. Binding by the enzyme of all three epoxides, measured by difference spectroscopy (Fig. 2), was clearly established by the observation in each instance of a type I binding spectrum. In each instance the difference spectrum, obtained im-

[4] P. R. Ortiz de Montellano and K. L. Kunze, Self-catalyzed inactivation of cytochrome *P*-450 by ethynyl substrates. *J. Biol. Chem.* (in press).

TABLE 4

Evaluation of NADPH-dependent cytochrome P-450 destruction by epoxides

The incubation procedure is summarized in the experimental section. The concentration of the substrate in each experiment is given in parentheses. The loss of cytochrome *P*-450 due to lipid peroxidation, measured as enzyme loss in the absence of substrate, is given as the first entry. This value, the average of seven experiments, is typical. The other entries are the averages of at least three determinations. The values cited for cytochrome *P*-450 loss in the presence of NADPH are those obtained after subtracting the loss due to lipid peroxidation (first entry is a typical value) from the total measured loss.

Substrate	NADPH (1 mM)	Percentage cytochrome *P*-450 loss		
		10 min	20 min	30 min
No substrate	Yes	1 ± 1	1 ± 1	2 ± 3
1,2-Epoxyheptane (10 mM)	Yes	5 ± 1	7 ± 1	7 ± 1
	No	4 ± 1	5 ± 1	6 ± 2
1-Heptene				
10 mM	Yes	28 ± 7	34 ± 7	38 ± 6
	No	11 ± 6	17 ± 3	21 ± 6
1 mM	Yes	10 ± 4	13 ± 3	14 ± 2
	No	0	0	0
4-Ethyl-1,2-epoxyhexane				
10 mM	Yes	6 ± 4	8 ± 4	9 ± 3
	No	1 ± 1	1 ± 2	1 ± 2
1 mM	Yes	2 ± 0	4 ± 1	5 ± 1
	No	0	0	0
4-Ethyl-1-hexene				
10 mM	Yes	22 ± 3	37 ± 6	46 ± 6
	No	2 ± 2	6 ± 6	8 ± 6
1 mM	Yes	18 ± 3	23 ± 1	27 ± 1
	No	0	0	0

mediately upon the addition of substrate, was stable with time. Evidence for this stability is provided by the linearity of the plots used to determine K_s values (Fig. 2). The difference spectra thus reflect binding of the epoxides themselves rather than of their metabolites. The apparent spectral binding constants (K_s) determined from the data in Fig. 2 for methyl 2-isopropyl-4,5-epoxypentanoate and 4-ethyl-1,2-epoxyhexane were 8 and 2 mM, respectively. The K_s value for 1,2-epoxyheptane, obtained from a similar spectral analysis (not shown), is 0.15 mM. These binding studies unequivocally demonstrate that the three epoxides are bound by the enzyme and that the available binding sites are saturated at the 10 mM concentration used to assay cytochrome *P*-450 destruction.

DISCUSSION

Evidence has been presented in a previous report that 2-isopropyl-4-pentenamide and its methyl ester analogue destroy cytochrome *P*-450 by a common mechanism in which the prosthetic heme is converted into an abnormal pigment by covalent attachment of the substrate (12). The present isolation of essentially identical pigments from rats treated with analogues of 2-isopropyl-4-pentenamide in which the amide is replaced by methyl ketone and methyl ether functions firmly establishes that a similar mechanism governs the previously noted destruction of cytochrome *P*-450 by these substrates (11). The activity of the methyl ether clearly contradicts the mandatory participation of a carbonyl group in the destructive process, but leaves open the possibility that a distal heteroatom, and not specifically a carbonyl, is required. Strong evidence that this is not so is provided by the observation that 4-ethyl-1-hexene and 1-heptene also destroy the enzyme (Table 1), that this destruction is NADPH dependent, and that it also results in the formation of abnormal hepatic pigments spectroscopically indistinguishable from those obtained with the oxygen-substituted substrates (Table 2). This evidence is buttressed by the failure of 3-ethylhexane and heptane to destroy the enzyme, a result consistent with the previously documented importance of the carbon–carbon double bond in the destructive action of homoallylic carbonyl structures (3, 5, 12). However, this evidence, although persuasive, is not beyond question, since it can be argued that the olefins are first hydroxylated by the monooxygenase at a position distal to the olefinic bond, and that only as the result of a subsequent catalytic event is the enzyme destroyed. To unambiguously challenge this interpretation, the interaction of cytochrome *P*-450 with ethylene, an olefin in which distal hydroxylation is patently impossible, was investigated. In effect, cytochrome *P*-450 is efficiently destroyed *in vitro* by pure ethylene but not by pure ethane (Table 3). Furthermore, this NADPH-dependent process results *in vivo* and *in vitro* in the accumulation of an abnormal hepatic pigment whose absorption spectrum shows that it is closely re-

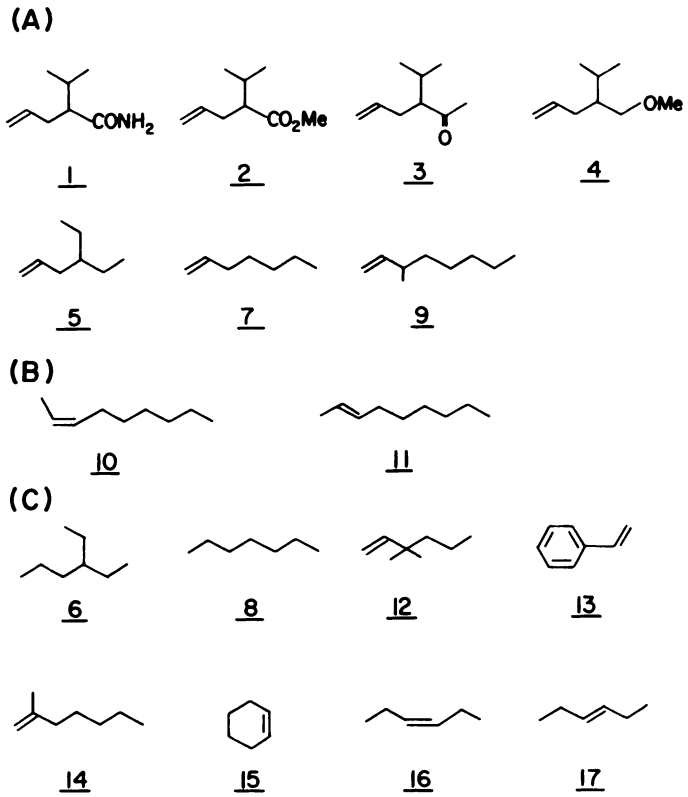

FIG. 1. *Structure of the substrates in Table 1*

(A) Substrates which cause NADPH-dependent cytochrome *P*-450 loss and which give abnormal ("green") hepatic pigments, (B) substrates which cause NADPH-dependent cytochrome *P*-450 loss but give no detectable hepatic pigments, and (C) substrates which do not cause significant NADPH-dependent loss of cytochrome *P*-450.

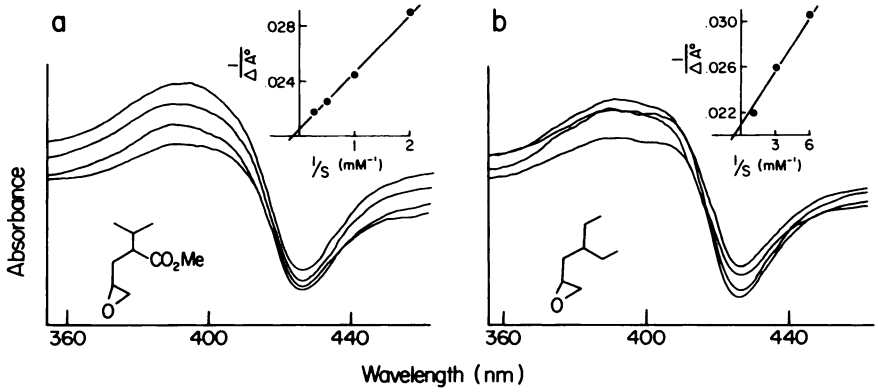

FIG. 2. *Difference spectra due to binding of epoxides to microsomal cytochrome P-450*

Difference spectra due to increasing concentrations of (a) methyl 2-isopropyl-4,5-epoxypentanoate and (b) 4-ethyl-1,2-epoxyhexane. Plots of the peak-to-trough absorbance difference against the reciprocal of the epoxide concentration, from which K_s values were determined, are given in the corresponding insets. The spectra were obtained on an Aminco–Chance DW-2 instrument as described in the literature (18), using hepatic microsomes from phenobarbital-pretreated rats. The assay mixture contained the following: microsomal protein (1 mg/ml), KCl (150 mM), and EDTA (1.5 mM) in 0.1 N phosphate buffer (pH 7.4). Epoxides were added without solvent with a precision syringe. Similar data (not shown) were obtained with 1,2-epoxyheptane.

lated to the pigments obtained with other substrates (Table 2). It is evident that the potential to interact destructively with cytochrome *P*-450 is an inherent property of carbon–carbon double bonds and that no other substrate feature is mechanistically essential to the process, although it remains possible, of course, that in favorable circumstances other functionalities in the substrate may become involved.

The presence of a double bond in the substrate, however, is not sufficient to guarantee cytochrome *P*-450 destruction. An instructive contrast, for example, is delineated by the relative activities of 1-heptene and 3-methyl-1-octene, both of which inactivate cytochrome *P*-450, and 3,3-dimethyl-1-hexene, which does not. These closely related structures effectively differ only in the degree of substitution at the carbon adjacent to the double bond, a fully substituted allylic position apparently interfering with the destructive interaction. One possible explanation for this result is that enzymatic manipulation of an allylic proton is critical for the destructive event. However, unless the destructive mechanism of ethylene is an exception, an allylic proton can not be required. It is therefore likely that the steric encumbrance of a highly substituted allylic position is responsible for attenuating (or suppressing) the destructive interaction. Allylic steric congestion is apparently more critical in the destruction of cytochrome *P*-450 by olefinic than acetylenic substrates, since vicinal trisubstitution has little effect on the destructive activity of acetylenes[4] (17, 20).

The presence of two, rather than one (or no), substituents on a double bond also interferes with enzyme destruction. The inactivity of 2-methyl-1-heptene and cyclohexene bears witness to this effect. Significant destruction is nevertheless mediated by *cis*- and *trans*-2-nonene, although it is not clear if this destruction is mechanistically related to that observed with terminal olefins since abnormal hepatic pigments have not been found with the internal olefins (Table 1). The marginal activity observed with 2-nonene, in which the double bond is one carbon atom away from the end of the chain, is not detectable with *cis*- and *trans*-3-hexene, in which the double bond is displaced inward by one more carbon. A striking analogy exists between the destruction of cytochrome *P*-450 by internal olefins and by internal (disubstituted) acetylenes, since the latter apparently also do not yield detectable abnormal hepatic pigments.[4] A clearer understanding of the relationship between enzyme destruction by terminal and nonterminal π-bonds must await the results of further experimental exploration.

We have recently reported that methyl 2-isopropyl-4,5-epoxypentanoate, the possible epoxide metabolite of methyl 2-isopropyl-4-pentenoate, did not destroy cytochrome *P*-450 and was therefore not an intermediate in the destructive process (12). This conclusion has now been strengthened by the demonstration that the epoxide readily binds to the enzyme under the experimental conditions utilized (Fig. 2). Parallel evidence has also been obtained which shows that epoxide metabolites play, at best, a minor role in the destruction of cytochrome *P*-450 mediated by other olefins. The marginal activity of 4-ethyl-1,2-epoxyhexane measured at a 10 mM concentration of the drug is undetectable at a 1 mM concentration even though the parent olefin is still highly active at the lower concentration. The marginal action of 1,2-epoxyheptane is also inadequate to account for the destructive activity of 1-heptene. The demonstration that a major fraction of these epoxides survives prolonged incubation with hepatic microsomes, and that 4-ethyl-1,2-epoxyhexane and 1,2-epoxyheptane bind to the enzyme active site with K_s values of 2 and 0.15 mM, clearly establishes that lack of activity is not due to rapid epoxide deactivation or exclusion from the active site. In sum, the present results leave no doubt that alkylation of cytochrome *P*-450 heme, the event which leads to enzyme destruction and abnormal pigment formation (10, 12), does not involve intervention of epoxide metabolites.

The identical molecular weights of the pigments produced by 2-isopropyl-4-pentenamide and its methyl ester analogue, which imply that the carbonyl groups of the substrates no longer differ in the isolated porphyrin adducts, suggested possible carbonyl group participation in the destructive mechanism (12). The present results

do not absolutely exclude such participation, but they do show that if it occurs it is not general. An alternative, and in view of our present results, more direct rationale can be formulated to explain the absence of a difference between the amide and ester carbonyl groups in the adducts of the corresponding substrates with prosthetic heme. The observed molecular ion for both adducts is consistent with the summation: protoporphyrin IX (dimethyl ester) plus substrate plus an oxygen atom, less ammonia (for the amide) or methanol (for the methyl ester) (12). Introduction of a hydroxyl group during adduct formation, as suggested by the presence of an extra oxygen atom, could precede carboxyl group involvement. Subsequent chemical lactonization, which requires loss of ammonia from the amide and methanol from the methyl ester, would then yield adducts of the correct molecular weight by a mechanism which involves only the carbon–carbon double bond in the destructive (alkylative) event.

Destruction of cytochrome *P*-450 with abnormal pigment formation by olefins bearing no heteroatomic substituents has not previously been reported. Previous studies with unsaturated compounds have involved substrates bearing oxygen, nitrogen, or halide substituents (for example, Refs. 21–23) and either have not searched for or have failed to find abnormal hepatic pigments. Vinyl chloride is particularly interesting in this regard, since it destroys both cytochrome *P*-450 and hepatic heme but apparently does not form a detectable hepatic pigment (23). Furthermore, evidence has been presented that the epoxide of vinyl chloride is not involved in the destruction of cytochrome *P*-450 (23). The mechanistic relationship between the destruction of cytochrome *P*-450 by ethylene and the closely related vinyl chloride is thus an important but still unresolved issue. There is little doubt in any case, given the similarity of the isolated pigments (Table 2), that ethylene and the other terminal olefins, like 2-isopropyl-4-pentenamide (12), destroy cytochrome *P*-450 by prosthetic heme alkylation.

Substances previously shown to destroy cytochrome *P*-450 during catalytic processing have invariably been nonphysiological chemical agents. Persuasive evidence now exists, however, that ethylene, long established as a plant hormone (24), is also a physiological product of mammalian metabolism (25–27). It is therefore not unreasonable to suggest that cytochrome *P*-450 destruction by endogenously formed ethylene may be a significant process under particular conditions of physiological stress precipitated by drugs, disease, diet, or environmental factors. An exploration of this new dimension in substrate-mediated cytochrome *P*-450 destruction is planned.

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