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INACTIVATION OF HEPATIC CYTOCHROME P-450 BY ALLENIC SUBSTRATES

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SUMMARY Hepatic microsomal cytochrome P-450 from phenobarbital-pretreated rats is destroyed by 17-α-propadienyl-19-nortestosterone, 1-propadienylcyclohexanol, and 1,1-dimethylallene. Substantial activity is also exhibited by 1-propadienylcyclohexanol against the enzymes in microsomes from 3-methylcholanthrene pretreated rats. The destructive process requires NADPH, is not inhibited by glutathione, and is paralleled by equimolar loss of hepatic heme but not by in vivo accumulation of identifiable "green" pigments. Attempted metabolism of allenes can thus result in destruction of cytochrome P-450.

Cytochrome P-450 enzymes, particularly those forms induced by phenobarbital, are destroyed during metabolism of unsaturated carbon-carbon bonds in certain substrates. This destructive interaction, first reported with 2-isopropyl-4-pentenamide (1,2), has subsequently been shown to occur with other unsaturated structures including secobarbital (3,4), vinyl chloride (5-7), fluroxene (8), norethisterone (9,10), and a number of propargylic insecticide synergists (11,12). In fact, the destructive activity of gaseous ethylene (13) and acetylene (12,14), the quintessential olefin and acetylene, clearly demonstrates that the potential to destroy cytochrome P-450 is an intrinsic property of unsaturated bonds rather than a property conveyed by molecular placement or heteroatomic substitution.

The destruction of cytochrome P-450 by olefins and acetylenes requires NADPH and molecular oxygen, is inhibited by carbon monoxide but not by glutathione, and is accompanied by a parallel decrease in microsomal heme content (2,3,7,9, 12-16). Loss of the enzyme after in vivo administration of some, but not all, of the destructive agents is associated with accumulation of abnormal hepatic

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("green") pigments. We have isolated such pigments, for example, after destruction of cytochrome P-450 by monosubstituted, but not by disubstituted, acetylenes (12). Pigment formation, when observed, provides important mechanistic information since we have established that the pigments are 1:1 covalent adducts of destructive agents with protoporphyrin IX, the porphyrin framework of the cytochrome P-450 prosthetic heme group (10,12,17,18).

The chemical properties of allenes, a class of unsaturated carbon functionalities distinct from olefins and acetylenes, have made their pharmacological exploitation of interest (see for example references 19-22). Essentially nothing is known, however, about the interaction of allenes with hepatic monooxygenases or about the metabolism of these functional groups. We now report a study using three allenic substrates which reveals that, as with olefinic and acetylenic groups, allenic functions can bring about destruction of cytochrome P-450 enzymes attempting to metabolize them.

MATERIALS AND METHODS

Samples of 17-α-propadienyl-19-nortestosterone (1) were provided by Syntex Research (Palo Alto, Ca.) and by Sandoz (Hanover, N.J.); 1-propadienylcyclohexanol (2) was obtained from Dr. Alf Claesson (Upsalla, Sweden) (21), and 1,1-dimethylallene (3) was purchased from Pfaltz and Bauer Co. These allenes were shown to be at least 99% pure by thin layer and gas chromatographic analyses.

Incubations. Unstarved Sprague-Dawley male rats weighing 200-250 g were injected intraperitoneally once a day for four days with either sodium phenobarbital (80 mg/kg, aqueous solution) or 3-methylcholanthrene (20 mg/kg as a 5 mg/ml solution in corn oil). The rats were sacrificed 24 hr after the last dose of inducing agent and hepatic microsomal preparations were obtained as previously reported (3,18). Incubations contained the following in addition to allenic substrates (1 mM): microsomal protein (1 mg/ml), NADPH (1 mM), KCl (150 mM), and EDTA (1.5 mM), all in 0.1 M Na/K phosphate buffer (pH 7.4). The incubation procedure and the assay for cytochrome P-450 content have been reported (12,18). Control incubations in the absence of NADPH, to measure NADPH-independent cytochrome P-450 loss, and in the absence of substrate, to measure substrate-independent (lipid-peroxidative) loss, were performed with each set of experiments. As long as unstarved rats were used, peroxidative and NADPH-independent enzyme losses after 20 min^{-1} of incubation did not exceed 2%. An extinction coefficient of 100 cm^{-1} mM^{-1} was used to calculate molar cytochrome P-450 losses (27). Except where indicated otherwise, the values in the tables are the average of at least three independent determinations. Standard deviations are given where appropriate.

Difference Binding Spectra. Binding of allenes to oxidized microsomal cytochrome P-450 from phenobarbital-pretreated rats was examined by difference spectroscopy (13,23) using an Aminco-Chance DW-2 instrument.

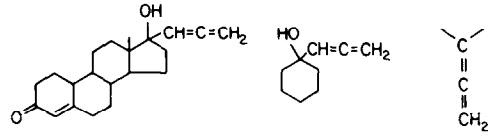
Pigment Isolation. Groups of three phenobarbital-induced rats were injected intraperitoneally (200 mg/kg) with either steroidal allene 1 (dose dissolved in 1 ml propylene glycol), 1-propadienylcyclohexanol (in 0.1 ml ethanol), or 1,1-dimethylallene (in 0.1 ml ethanol). The rats were sacrificed four hours later and their livers were extracted as previously described (10, 12,13). The characteristic green (red-fluorescing) bands we have observed with other agents (10,12,13) were not detectable on thin layer chromatographic analysis of the extracts except for a faint band in the experiment with 1,1-dimethylallene.

Microsomal Heme Determinations. Small (1 ml) aliquots drawn from incubation mixtures with and without NADPH were diluted with 2 ml of a 20% pyridine in 0.1 N NaOH solution. Heme content in each aliquot was calculated from the 557-600 nm absorbance difference between dithionite reduced and unreduced samples, using a value of 34.4 cm^{-1} mM^{-1} for the extinction coefficient (24,27). Values given are the average of three determinations.

RESULTS AND DISCUSSION

Incubation of allenic substrates 1-3 (Fig. 1) with microsomes from phenobarbital pretreated rats resulted in rapid loss of spectroscopically-measured cytochrome P-450 (Table 1). Enzyme loss was completely dependent on the presence of NADPH, suggesting that catalytic participation of the enzyme was required for its destruction. Catalytic participation of the enzyme has been confirmed in studies to be published using purified, reconstituted cytochrome P-450.[1] Lipid-peroxidative enzyme loss was excluded by the fact that no more than 2% of the enzyme was lost if a substrate was not added to the incubation mixture, so long as microsomes were obtained from unstarved rats. Furthermore, as shown in Table 1, addition of 5 mM glutathione did not moderate the destructive activity of the allenic steroid, a result which argues against the intervention of a diffusible, electrophilic metabolite in the destructive process.

Binding of 1-propadienylcyclohexanol and 1,1-dimethylallene (Fig. 2) to the enzyme could be demonstrated by difference spectroscopy (23), although an analogous Type I binding spectrum was not obtained with the allenic steroid.



1 2 3

Fig. 1. Structure of allene substrates.

TABLE 1. Destruction by allenes of cytochrome P-450 enzymes in microsomes from phenobarbital (Pb) and 3-methylcholanthrene (MC) induced rats.

Substrate (1 mM)	Inducer	NADPH (1 mM)	Percent P-450 destruction at given time: 10 min	20 min
1	Pb	Yes	43±5	57±6
		Yes[a]	--	55±4
		No	0	0
2	Pb	Yes	29±3	35±3
		No	0	0
3	Pb	Yes	28±4	52±2
		No	0	0
1[b]	MC	Yes	8	8
		No	0	0
2	MC	Yes	25±3	36±2
		No	0	0
3[b]	MC	Yes	1	3
		No	0	0

[a]Glutathione (5 mM) was also added to this incubation.

[b]These experiments were only done in duplicate. The two values differed by less than 1% in each instance.

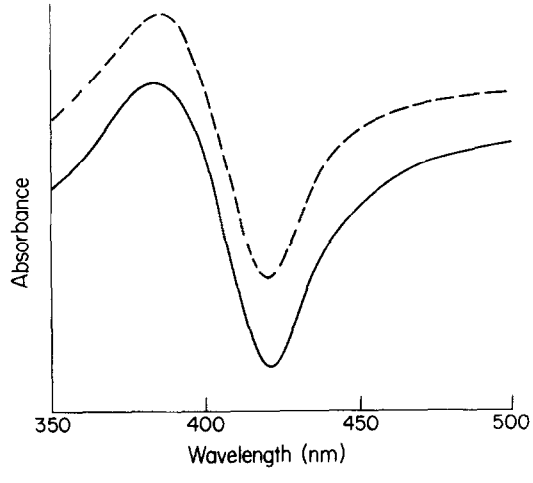


Fig. 2. Difference spectrum caused by addition of 2 and 3 to microsomes from phenobarbital-pretreated rats. The lower curve was obtained with 2 and the upper curve with 3 (the baseline is displaced so that the two curves can be clearly distinguished).

The absence of a defined binding spectrum with the steroid is reminiscent of the absence of such a spectrum with 2-isopropyl-4-pentenamide (allylisopropylacetamide), a compound whose binding can nevertheless be demonstrated by competition experiments (25). The reason for this absence of a binding spectrum remains to be established.

In contrast to the marked destruction of the phenobarbital-inducible enzyme by all three allenes, only 1-propadienylcyclohexanol exhibited unambiguous activity against cytochrome P-450 enzymes in microsomes from 3-methylcholanthrene treated rats (Table 1). The marginal decrease brought about by the steroidal allene may only reflect destruction of minor forms of cytochrome P-450 present in 3-methylcholanthrene induced microsomes. In any case, the data in Table 1 clearly show that phenobarbital-inducible forms of cytochrome P-450 are particularly susceptible to destruction by allenes. On the other hand, the activity of 1-propadienylcyclohexanol in both types of microsomes places it in the same class as fluroxene, which also is characterized by this ambidextrous activity.

A close quantitative relationship exists between independently measured *in vitro* loss of phenobarbital-inducible cytochrome P-450 and microsomal heme (Table 2). The 1:1 molar ratio of these two processes, particularly in view of the *in vitro* protocol which avoids some of the ambiguities of analogous *in vivo* studies, strongly suggests that they are mechanistically linked. However, despite a search using methodology currently employed in this laboratory (10,12,13,18), abnormal heme-derived pigments were not detected in the livers

TABLE 2. Relationship between loss of phenobarbital-inducible cytochrome P-450 and loss of microsomal heme.

Substrate	P-450 loss (nmoles)	Heme loss (nmoles)	Loss ratio P-450/heme
1	683±91	650±43	1.0/0.95
2	487±41	473±45	1.0/1.0
3	693±50	606±130	1.0/0.9

of rats treated in vivo with any of the allenic substrates. Although a trace of such a pigment was observed after treatment with 1,1-dimethylallene, too little of it was present to isolate and characterize. Its artifactual formation can not therefore be excluded.

The destruction of cytochrome P-450 by the three allenes requires catalytic turnover of the enzyme, is selective for specific enzyme forms,is not attenuated by glutathione, is matched by an equimolar loss of microsomal heme, but does not result in formation of abnormal hepatic pigments similar to those obtained with terminal olefins and acetylenes. The destructive process thus most closely resembles that mediated by internal acetylenes, which also do not cause formation of hepatic pigments (12). In the absence of such pigments the destructive event can not be defined, although the data are consistent with participation of a reactive species formed during oxidative metabolism of the allene functionality. The precedent set by our studies with acetylenes suggests an exo-methylene epoxide, the highly unstable product obtained on chemical oxidation of an allene (26), or a transient species formed during the enzymatic epoxidation sequence, as the possible reactive agent, although a distinction between the heme and apoprotein as reaction targets can not now be made. It is nevertheless clear, from the lack of dependence of the destructive process on structural features other than the presence of an allene functionality and from the inactivity of closely related saturated systems (9,13), that cytochrome P-450 is destroyed during catalytic interaction with allenic functionalities.

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