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SUICIDAL INACTIVATION OF HEPATIC CYTOCHROME P-450 IN VITRO BY SOME ALIPHATIC OLEFINS

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SUMMARY: Vinylalicyclic olefins destroyed hepatic cytochrome P-450 from phenobarbital-pretreated male mice, in the presence of NADPH; the loss of the enzyme was always accompanied by a quantitatively correlated loss of microsomal heme. The same compounds caused much smaller losses of cytochrome P-450 and heme in 3-methylcholanthrene-microsomes than with PB-ones. The effect has been shown to be specific for cytochrome P-450, it is not stimulated by NADH, it is inhibited by CO, Methyrapone, and it shows pseudo first-order kinetics. The epoxides of the parent active olefins did not intervene in the destruction of the enzyme. No cytochrome P-450 loss was observed after incubation with cyclohexene, 1,3-and 1,4-cyclohexadiene, and isopren.

Hepatic microsomal cytochrome P-450 and particularly the PB-inducible isozymes are destroyed during the metabolism of certain unsaturated substrates (1,2). The destruction of cytochrome P-450 by allylisopropylacetamide (AIA) was the first observed (3,4) and the most studied of these destructive interactions (5), that have been observed later with secobarbital (2), fluroxene (6), vinyl chloride (7), some olefins (8) and acetylenes (9,10). The inactivation of cytochrome P-450 requires NADPH and molecular oxygen, is inhibited by carbon monoxide and SKF-525A and there is a link between the loss of cytochrome P-450 and of microsomal heme (3,4,7,8). In fact destruction of the enzyme results in conversion of the prosthetic heme group into a green porphyrin that is a 1:1:1 covalent adduct of the substrate, protoporphyrin IX and oxygen atom (11-14). Since both ethylene (8) and acetylene (9) are destructive agents, the inactivation of cyt.P-450 would seem to depend only on the presence of a carbon-carbon double or triple bond, but some chemicals with unsaturated sites (8) cause no cytochrome P-450 losses. Thus, in an attempt to get an insight on the structural requirements for the destructive event, we are

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now reporting a study on cytochrome P-450 in vitro destruction by some aliphatic olefins and their corresponding epoxides.

MATERIALS AND METHODS

Chemicals: Vinylcyclopentane was obtained from EGA; the other olefines and epoxides were from Fluka. The epoxyethyl cyclopentane, the epoxyethyl cyclohexane and the epoxyethyl cyclooctane were obtained by 3-chloro peroxybenzoic acid (Fluka) epoxidation of the corresponding olefins in dry CH_2Cl_2 (15). Epoxide were checked by g.l.c.. The epoxyethyl cyclopentane boiled at 150°C/760 mm Hg- IR 1240 cm^{-1}. The epoxyethyl cyclohexane distilled at 70°C/5 millibar, reported bp 63-65°C/14 mmHg (16) - IR 1240 cm^{-1}. The epoxyethyl cyclooctane distilled at 90°C/4 millibar - IR 1240 cm^{-1}. All liquid samples were distilled prior to use. All other chemicals were analytical grade reagents.

Treatment of animals and isolation of microsomal fraction: Unstarved Swiss albino male mice (6-9 weeks old), weighing 22-35 g were injiected i.p. once a day for 3 or 4 days with either sodium phenobarbital (PB) (100 mg/Kg, acqueous solution) or 3-methylcholanthrene (3-MC) (80 mg/Kg in corn oil). The animals were sacrificed 24 hours after the last dose of inducing agent and hepatic microsomal preparations were obtained as previously described (17), except that phosphate buffer was substituted by 50 mM Tris-HCl buffer (pH 7.4) (18). The protein concentration was measured by the Lowry procedure (19), using bovine serum albumin as a standard.

In vitro cytochrome P-450 destruction assay: Reaction mixtures contained the following: microsomal protein (2 mg/ml); $NADP^+$ (0.433 mM), $MgCl_2$ (3.3 mM), glucose-6-phosphate (5.46 mM)in 50mM Tris-HCl buffer (pH 7.4), containing 1.15% KCl and EDTA (1 mM). The incubation (5 ml) were initiated by addition of the substrate (1 M in CH_3OH solution) and glucose-6-phosphate-dehydrogenase (0.33U/ml) to the above mixture after 5 min preincubation period at 37°C. At the used concentration, methanol has no destructive interactions with cytochrome P-450. After incubation at 37°C in a shaking bath (140 strokes/min) in air for various time, the flasks were rapidly immersed in an ice-bath. In some experiments, where indicated, NADPH was substituted by NADH (1 mM), or both NADH (1 mM) and NADPH (1 mM) were added. In order to measure NADPH independent cytochrome P-450 loss, control incubations in the absence of NADPH were performed with each substrate. Each set of experiments included a lipid peroxidation control, in which an incubation was carried out, without addition of substrate. Only minor cytochrome P-450 losses were observed in these cases, indicating that lipid peroxidation was effectively suppressed under the experimental conditions used.

Cytochrome P-450 and b_5 concentrations were determined according to Omura and Sato (20) on a Perkin Elmer 576 ST spectrophotometer. Cytochrome P-450 losses reported in the text have been corrected for lipid peroxidation losses (0-5%); excepted where indicated otherwise all the values in the tables are average (± S.E.) of at least three independent determinations on different microsomal preparations. Cytochrome P-450 reductase activity was measured by its ability to reduce cytochrome c (21). Heme was determined as the pyridine hemochromogen (20).

RESULTS AND DISCUSSION

In vitro destruction of cytochrome P-450 from PB-pretreated mice: All the aliphatic olefins we have studied seem to be substrates

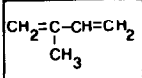
OLEFIN (1 mM)	% Loss of Cyt. P-450 NADPH (1 mM)	% Loss of microsomal heme NADPH (1 mM)
	0	8.1 ± 1.2
	0	10.7 ± 0.4
	0	11.8 ± 1.1
	0	10.5 ± 2.2
	19.3 ± 1.0	24.5 ± 1.0
	27.1 ± 2.5	37.1 ± 1.5
	26.4 ± 1.5	30.4 ± 2.1
	26.9 ± 3.6	31.3 ± 1.9
 (*)	26.3 ± 3.2	38.5 ± 2.2

Fig. 1. In vitro destruction of hepatic microsomal cytochrome P-450 from PB-pretreated male mice by olefins (1 mM) after 30 minutes of incubation at 37°C. In the absence of NADPH there was no loss of cyt.P-450 in all cases.(*) Olefin concentration 5 mM.
The procedure is given in the experimental section.
The values have been corrected for minor losses due to lipid peroxidation. Each value is the average of a minimum of three determinations and is given with its standard error.

for cytochrome P-450; their binding to the enzyme could be demonstrated by difference spectroscopy (22): a type I binding spectrum was obtained with all the olefins studied. (data not shown).

Figure 1 shows the ability of the considered olefins to cause cytochrome P-450 and heme losses in hepatic microsomal preparations from PB-pretreated mice, after 30 min of incubation at 37°C, when their nominal concentration in the reaction mixture was 1 mM. With four of the nine studied chemicals (isopren; 1,3-cyclohexadiene; 1,4-cyclohexadiene and cyclohexene) no substantial enzyme loss (<5%) was observed, both in the presence of NADPH and in its absence. The higher decrease of microsomal heme with respect to that of cytochrome P-450 in the presence of

NADPH is due to heme-oxygenase (23), a microsomal NADPH- and O_2-dependent enzyme, metabolizing the free heme, solubilized in microsomal membranes. Incubation of all the vinylalicyclic olefins resulted in enzyme and heme loss. This loss completely depended on the presence of NADPH, was selective for cyt.P-450, since neither cytochrome b_5 nor decrease in NADPH-cytochrome c-reductase activity was observed. The close parallelism observed between independently measured loss of cyt.P-450 and microsomal heme, strongly suggests that the two events are mechanistically linked and similar to those caused by AIA and other unsaturated substances. Among all the nine investigated olefins, only the vinylalicyclic ones destroy cyt.P-450 in the presence of NADPH. Conjugated and unconjugated dienes (isopren, 1,3-cyclohexadiene and 1,4-cyclohexadiene) as well as simple internal alicyclic olefins (cyclohexene) show no destructive activity on cyt.P-450. The loss of the enzyme is quantitatively similar for all vinylalicyclic olefins and rather independent from the structure of the cyclic residue bound to the vinyl group. The present results except for isopren agree with the theory, according to which terminal olefins (8) or acetylenes (24) are more effective in causing the loss of cyt.P-450 than compounds whose π bonds have an internal position. The conjugation of the terminal double bonds in isopren could be the cause of such lack of effect.

Substrate concentration, incubation time, cofactors and inhibitors: Dependence of cyt.P-450 destruction on the concentration of substrate at a fixed incubation time has been investigated with vinylcyclohexane (fig.2). Time dependence of the destructive event in the presence of different vinylcyclohexane concentrations was also investigated (Fig.3). This destructive process resulted to be saturated when the substrate concentration was 5 mM after 30 min of incubation: the cytochrome P-450 loss reached 45% of the total enzyme whose level was stable even after 60 min of incubation. A similar plateau was reached also with AIA (5). Thus, assuming that only 45% of the total enzyme in our microsomal preparations was available for inactivation, a 45% loss of enzyme was equated with 100% destruction.

In fig. 4 the percentage of enzyme available for destruction still present is plotted (in log scale) against incubation time for some concentrations of vinylcyclohexane. The apparent half life ($t_{\frac{1}{2}}$) of the enzyme at different concentrations of vi-

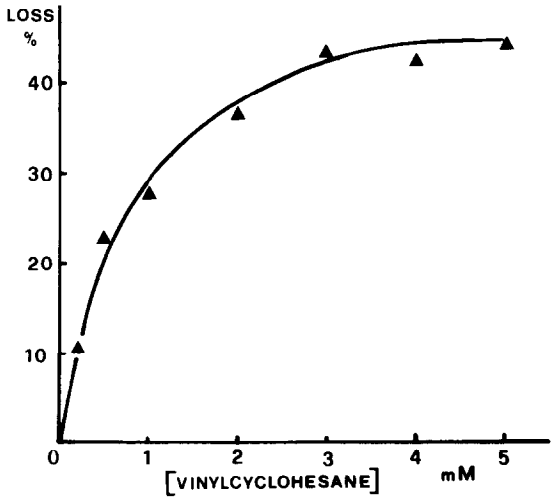


Fig. 2. Dependence of cytochrome P-450 destruction on concentration of vinylcyclohexane.
Incubation time was fixed at 30 min. Microsomes from PB-pretreated male mice were used. Each point is the average of two determinations.

nylcyclohexane can be calculated from fig.4. These values of half-life plotted against the reciprocal of the substrate concentration gave a linear relationship (fig.5), showing that a destruction event has pseudo-first order kinetics typical of suicidal enzymic process as reported with AIA (5). The half-

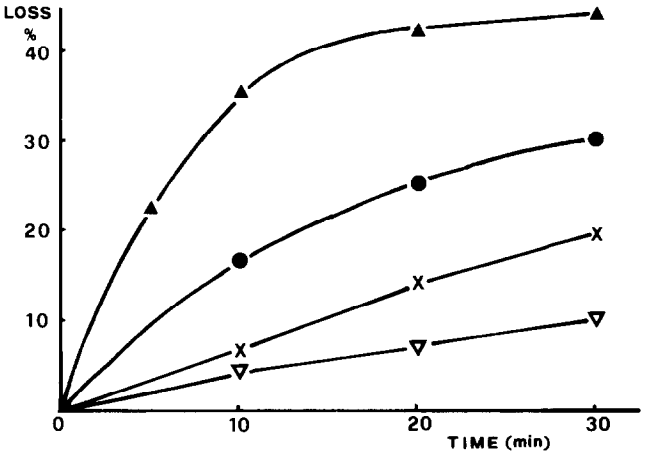
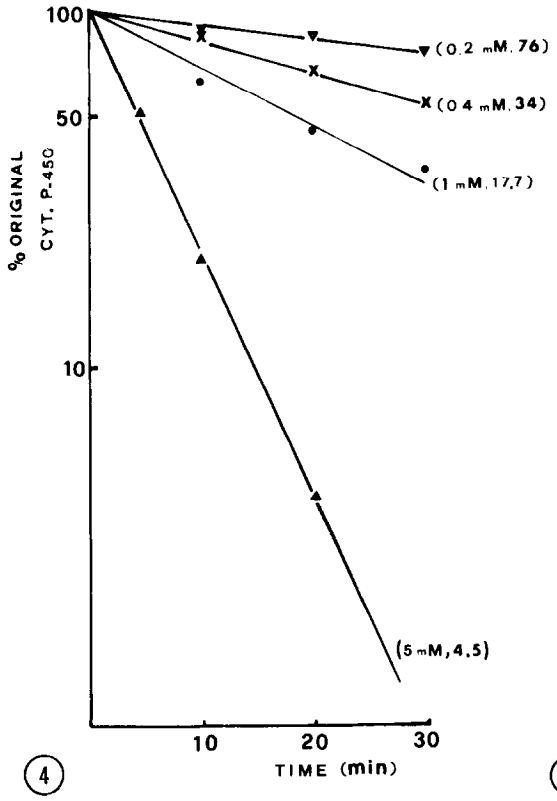


Fig. 3. Time dependence of cytochrome P-450 destruction.
Cytochrome P-450 loss after incubation of PB-microsomes in the presence of different vinylcyclohexane concentrations (▲, 5mM; ● 1mM; X 0.4mM; ▽ 0.2mM) for the indicated time is given. Each point is the average of two determinations.



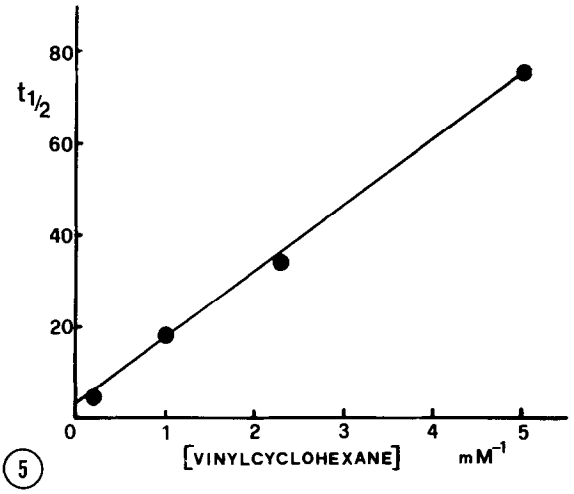


Fig. 4. Rates of cytochrome P-450 inactivation at various concentrations of vinylcyclohexane.
The percentage residual cytochrome P-450 (making equal to 100% the destructible enzyme), is plotted on a log scale vs. time of incubation at various vinylcyclohexane concentrations. For each concentration the corresponding half-life of the cytochrome P-450 destruction process is given in parenthesis.

Fig. 5. Kinetic analysis. The half-lives for the cytochrome P-450 inactivations by vinylcyclohexane calculated from fig.4 are plotted against the reciprocal of the corresponding substrate concentration.

life at the fastest inactivation rate gave a $t_{\frac{1}{2}}$ = 3.5 min with a constant rate of $3.3 \times 10^{-3} s^{-1}$ which resulted to be about double than that of AIA (5). The destruction of cytochrome P-450 was not stimulated by NADH (1mM) and addition to an incubation mixture containing NADPH (1mM) of 1 mM NADH did not increase enzyme loss after 30 min of incubation, at 37°C. Furthermore addition of 2 mM glutathione did not affect NADPH-dependent losses of cytochrome P-450 and heme. The destruction process by 5 mM vinylcyclohexane was completely abolished using classical monooxigenase inhibitors as CO and Methyrapone (0.2mM) while the SKF 525A at 0.2mM caused a 60% inhibition of cytochrome P-450 destruction.

OLEFIN (1 mM)	% Loss of Cyt. P-450 NADPH (1 mM)	% Loss of microsomal heme NADPH (1 mM)
	7.0 ± 2.5	17.9 ± 2.6
	18.7 ± 1.7	21.2 ± 1.2
	15.2 ± 2.0	19.7 ± 2.6
	0	10.5 ± 1.1

Fig. 6. Destruction of cytochrome P-450 from 3-MC-pretreated male mice by olefins (1mM) after 30 min of incubation at 37°C. In the absence of NADPH there was no loss of cyt. P-450 in all cases.
Other details are given in Figure 1.

In vitro destruction of cytochrome P-450 from 3-MC pretreated mice: In contrast to the marked destruction of the PB-inducible isozymes by all the five vinyl olefins, the loss of cytochrome P-450 was much reduced when hepatic microsomes from 3-MC-treated mice were used for the incubations (Figure 6). The destructive interaction was still NADPH-dependent and was parelleled again by heme loss. This result strongly suggests that olefins destroy particularly those forms of cytochrome P-450, which are able to metabolize them. This fact is strongly supported by studies with purified, reconstituted enzyme systems, demonstrating activity of AIA against the PB-isozymes, but no destructive effects with 3-MC-isozyme,which cannot metabolize the chemical agent(25).

Destructive effects of epoxides:

Although Ortiz de Montellano excluded the role of the epoxides corresponding to AIA (26) - and of few other olefins (8) - in the cyt.P-450 distruction, recently a possible involvement of an epoxide metabolite of an allyl compound has been reported(27). Thus the possible epoxide metabolites of our active olefins were synthetized and their interaction with hepatic cytochrome P-450 was investigated. The epoxides (epoxyethyl cyclopentane, epoxyethyl cyclohexane, epoxyethyl cyclooctane, epoxyethyl cyclohexene 3-4 oxide,epoxycyclohexane)were incubated with hepatic microsomes under the same experimental conditions as their parent

olefins. After 30 min of incubation a slight loss of cyt.P-450 and heme occurred only with epoxyethyl cyclohexane at concentration of 10 mM; this effect disappeared at lower concentration (5 mM). The presence of the epoxides at the end of incubation was checked by extracting with ether and assaying the epoxides with g.l.c.: in any case the recovery was greater than 50% of that initially present. The present results leave little doubt that cyt.P-450 destruction by vinylaliciclic olefins does not involve intervention of epoxide metabolites, and support the hypothesis that the prosthetic heme is alkylated by a reactive species generated in situ. The fact that addition of 2 mM glutathione did not moderate the losses of cyt.P-450, argues against the intervention of a diffusible, electrophilic metabolite in the irreversible inactivation of cyt.P-450.

The transient species could be produced either directly by the attack of electrophilic oxygen on the double bond-to give a zwitterion type intermediate without oxirane ring closure, in agreement with the currently accepted oxenoid nature of the active oxygen(28)-or by a proton catalyzed ring opening of the formed epoxide (29). The fact that styrene, whose epoxide is more susceptible than the presently investigated stable aliphatic epoxides (half-lives>24 hours in TRIS-HCl 50 mM , pH 7.4) to proton catalyzed ring opening, shows no destructive effects(8) on cyt.P-450, is more consistent with the former than with the latter hypothesis.

Thus the inability of the epoxides-related to our olefins-to destroy cytochrome P-450 and their stability strenthens the hypothesis that an intermediate zwitterion species is responsable for the destructive process before the oxirane ring formation.

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