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Effects of Linoleic Acid Hydroperoxide on the Hepatic Monooxygenase Systems of Microsomes from Untreated, Phenobarbital-Treated, and 3-Methylcholanthrene-Treated Rats

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SUMMARY

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Increasing concentrations of linoleic acid hydroperoxide (10-250 μM) destroyed increasing amounts of P-450 hemoprotein in hepatic microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats. Loss of P-450 hemoprotein in microsomes from untreated and phenobarbital-treated rats was biphasic; 50% was destroyed at low concentrations of linoleic acid hydroperoxide (50 μM or less), and only about half the remainder was destroyed at relatively high concentrations (150 μM or more). The labile population of P-450 hemoprotein was designated P-450$_{(II)}$, and the stable, P-450$_{(ls)}$. The loss of P-450 hemoprotein in microsomes from 3-methylcholanthrene-treated rats was not biphasic, and most of the hemoprotein was in the stable form. Almost all of the monooxygenase activity (ethylmorphine *N*-demethylase, aniline hydroxylase) and type I binding (hexobarbital) was associated with P-450$_{(II)}$. Microsomal NADPH-cytochrome *c* reductase activities and cytochrome b_5 levels were not affected by linoleic acid hydroperoxide until high concentrations were reached (150 μM or more). NADH-cytochrome *c* reductase activity was similarly unaffected by linoleic acid hydroperoxide in microsomes from untreated rats, was slightly elevated in microsomes from 3-methylcholanthrene-treated rats, and was greatly elevated in microsomes from phenobarbital-treated rats. NADPH oxidase activity was unaffected by linoleic acid hydroperoxide in microsomes from untreated and 3-methylcholanthrene-treated rats, but was decreased slightly in microsomes from phenobarbital-treated animals. These results led to the conclusion that P-450$_{(II)}$ functions in the oxidation of exogenous substrates, that P-450$_{(ls)}$ functions in the oxidation of endogenous substrates, and that the two functions are essentially independent of each other.

INTRODUCTION

NADPH-dependent lipid peroxidation interferes with hepatic microsomal monooxygenase reactions (1). This is thought to occur in at least three ways: (a) by disrupting the microsomal membrane (2), (b) by competing for reducing equivalents through a common electron transfer sys-

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tem (1), and (c) by destroying the heme of P-450 hemoproteins[1] (3). The number and diversity of these mechanisms greatly complicate the interpretation of studies of adverse effects of peroxidation on monooxygenase systems. The observation of Hrycay and O'Brien (3) that synthetic linoleic acid hydroperoxide destroys P-450 hemoproteins and lowers monooxygenase activity in microsomes presented an opportunity to study the effects of the immediate products of the peroxidase reaction on the monooxygenase system without introducing those complicating factors which occur when the lipid peroxides are generated in the microsome enzymatically. Evidence is presented to show that two types of P-450 hemoprotein exist in microsomes, one which is highly susceptible to destruction by linoleic acid hydroperoxide, and one which is quite resistant. Most of the monooxygenase activity of microsomes is associated with the labile hemoprotein. This communication describes the effects of linoleic acid hydroperoxide on microsomal P-450 and b_5 hemoproteins, NADPH- and NADH-cytochrome *c* reductases, NADPH oxidase, ethylmorphine *N*-demethylase, aniline hydroxylase, and the spectral binding of hexobarbital and aniline.

MATERIALS AND METHODS

Animals. Male Holtzman rats (180–220 g) were injected intraperitoneally with NaCl, sodium phenobarbital (40 mg/kg/day), or 3-methylcholanthrene in corn oil (20 mg/kg/day) for 4 days and killed 24 hr after the last injection.

Microsomal preparations. Hepatic microsomes, prepared as described previously (4), were suspended in a solution of 1.15% KCl and 0.1 M phosphate buffer, pH 7.4, to a protein concentration of 5 mg/ml. Amounts of linoleic acid hydroperoxide were added to these suspensions to give graded concentrations between 0 and 250 μM. The preparations were allowed to stand at room temperature for 20 min, after which reduced glutathione (final concentration, 2.0 mM) was added to remove unreacted linoleic acid hydroperoxide. Appropriate dilutions of untreated and linoleic acid hydroperoxide-treated microsomes were made with buffer solution. All preparations were used on the day the animals were killed.

Assays. Ethylmorphine *N*-demethylase and aniline hydroxylase activities of microsomal preparations were determined as described previously (5), except that nicotinamide was omitted. The procedures of Nash (6) and of Imai *et al.* (7), respectively, were used to estimate rates of formaldehyde and *p*-aminophenol formation. The concentrations of substrate were 2 and 0.2 mM for ethylmorphine *N*-demethylase and aniline hydroxylase assays, respectively; the concentration of microsomal protein was 1 mg/ml in both assays. The incubation media contained EDTA (0.2 mM) to prevent lipid peroxidation and minimize nucleotide pyrophosphatase activity (8). NADPH oxidase activity was determined as described previously (8). P-450 and b_5 hemoproteins were determined as described by Omura and Sato (9). NADPH- and NADH-cytochrome *c* reductase activities were measured by a modification of the method of Phillips and Langdon (10): 0.33 mg of microsomal protein and 50 μM cytochrome *c* in 0.1 M phosphate buffer, pH 7.4, were warmed to 37°, and the increase in absorbance at 550 nm was recorded after the addition of 0.83 mM NADPH or NADH. Cyanide was omitted (11). Microsomal heme was determined by the hemochromogen method of Paul *et al.* (12) after treating the microsomes with steapsin to remove cytochrome b_5 (13).

Substrate binding spectra were determined as described previously (5), using a concentration of microsomal protein of 1 mg/ml and concentrations of aniline and hexobarbital of 10 and 30 mM, respectively. All spectral measurements were made with an Aminco DW-2 spectropho-

[1] Throughout this communication, P-450 hemoprotein is considered to be any hemoprotein occurring in hepatic microsomes which, when reduced and combined with carbon monoxide, gives a difference spectrum with a maximum absorption at or near 450 nm. Examples of individual or classes of P-450 hemoproteins are cytochrome P-450, the predominant P-450 hemoprotein in hepatic microsomes from untreated rats, and cytochrome P_1-450 (or P-448), the predominant P-450 hemoprotein found in hepatic microsomes from rats treated with 3-methylcholanthrene.

tometer. Protein was determined by the method of Lowry *et al.* (14).

Chemicals. O'Brien's methods (15) were used for synthesizing and quantifying linoleic acid hydroperoxide. The quantification procedure, which employs an extinction coefficient for linoleic acid hydroperoxide of 25.25 mM^{-1} cm^{-1} at 233 nm, was verified with a radioassay by mixing 50 μCi of [1-^{14}C]linoleic acid (New England Nuclear; 50 Ci/mole) with 5 g of carrier linoleic acid (Sigma), which was then processed by the method of O'Brien to yield purified labeled linoleic acid hydroperoxide. Radioactive and ultraviolet assays were in good agreement. Linoleic acid hydroperoxide was shown by both procedures to be stable for at least 2 weeks when stored in ethanol at −10°.

Glutathione was obtained from Sigma. The sources of other chemicals and enzymes have been given previously (5).

RESULTS

Time course of destruction of P-450 hemoprotein by linoleic acid hydroperoxide and termination of reaction with glutathione. For the studies we had in mind, it was first necessary to determine the time course of destruction of P-450 hemoprotein by linoleic acid hydroperoxide and to establish that excess hydroperoxide could be destroyed at will with glutathione. The destructive effect of 113 μM linoleic acid hydroperoxide on P-450 hemoprotein was terminated by glutathione regardless whether it was added at 0, 8, 15, or 20 min after the addition of the hydroperoxide (Fig. 1). Figure 1 also shows that the maximal effects of both the lower (113 μM) and the higher (225 μM) concentrations of linoleic acid hydroperoxide were achieved within the 20-min incubation period at room temperature.

O'Brien and Rahimtula (16) showed that lipid hydroperoxides induce lipid peroxidation in hepatic microsomes and provided indirect evidence that this reaction is mediated by cytochrome P-450. Their data were derived from studies with cumene hydroperoxide, but they stated that linoleic acid hydroperoxide is an even more potent inducer of lipid peroxidation. The possibility was therefore considered that in our studies the loss of P-450 hemoprotein might have been due in part to linoleic acid hydroperoxide-induced peroxidation of membrane lipids. This proved not to be the case. O'Brien and Rahimtula showed that 0.1 mM Mn^{++} completely inhibited lipid peroxide-induced lipid peroxidation in hepatic microsomes. The addi-

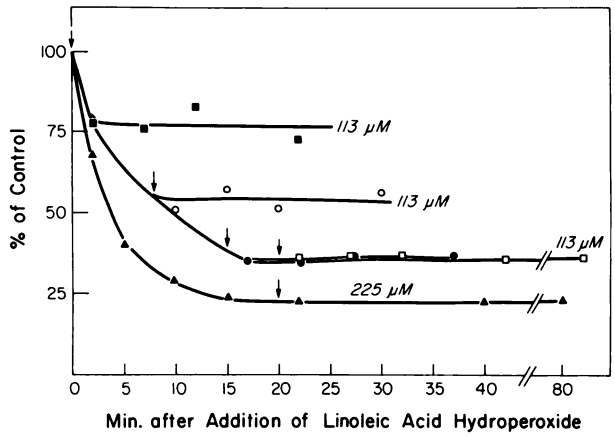

FIG. 1. *Destruction of P-450 hemoprotein by linoleic acid hydroperoxide and its termination by glutathione*
Linoleic acid hydroperoxide (113 or 250 μM) was added to suspensions of hepatic microsomes in 0.1 M phosphate buffer (5 mg of protein per milliliter) at room temperature. Samples were taken at selected time intervals, diluted to 1 mg of protein per milliliter with phosphate buffer, and assayed for P-450 hemoprotein content. Glutathione (2.0 mM) was added (as shown by the arrows) 0, 8, 15, or 20 min after the addition of 113 μM linoleic acid hydroperoxide, or 20 min after 225 μM hydroperoxide. P-450 hemoprotein assays were performed 2, 7, 12, and 22 min after the addition of glutathione.

tion of 0.1 mM Mn^{++} to microsomes prior to the addition of linoleic acid hydroperoxide (150 μM) had no effect on the loss of P-450 hemoprotein from microsomes from untreated rats (data not shown).

When linoleic acid (75 or 150 μM) was substituted for linoleic acid hydroperoxide, no loss of microsomal P-450 hemoprotein or ethylmorphine *N*-demethylase was observed (data not shown).

Effect of temperature on destruction of P-450 hemoprotein by linoleic acid hydroperoxide. Figure 2 shows the effect of temperature on the time course of destruction of P-450 hemoprotein by 250 μM linoleic acid hydroperoxide. The initial rates of destruction were not greatly affected by differences of temperature between 0° and 37°, and the extent of destruction at the end of 20 min did not vary greatly with temperature.

Effect of concentration of linoleic acid hydroperoxide on P-450 and b_5 hemoproteins. Figure 3 shows the destructive effect of increasing concentrations of linoleic acid hydroperoxide on hemoproteins of hepatic microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats. The disappearance of

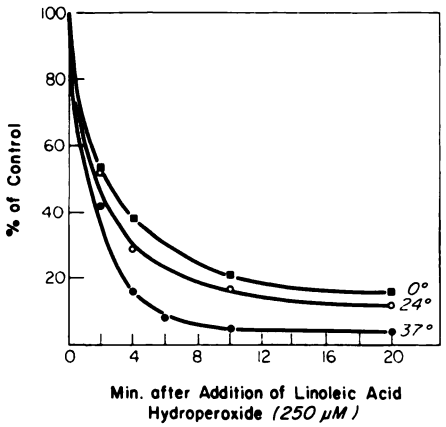

FIG. 2. *Effect of temperature on destruction of P-450 hemoprotein by linoleic acid hydroperoxide*

Hepatic microsomes were incubated for 20 min with linoleic acid hydroperoxide (250 μM) at 0°, 24°, or 37° as described in Fig. 1, and glutathione (2.0 mM) was added at the end of the incubation period. P-450 hemoprotein assays were performed at selected time intervals after the addition of the hydroperoxide.

P-450 hemoprotein in microsomes from untreated animals was biphasic; about 50% of the P-450 hemoprotein was destroyed at a concentration of linoleic acid hydroperoxide of 50 μM or less, and only half the remaining hemoprotein was destroyed at a concentration of 150 μM or more. To facilitate discussion of the types of P-450 hemoprotein represented by these two phases, we employ $P\text{-}450_{(II)}$ to designate the hemoprotein which is relatively labile at low concentrations of linoleic acid hydroperoxide, and $P\text{-}450_{(ls)}$ to designate the hemoprotein which is relatively stable at low concentrations of linoleic acid hydroperoxide. We emphasize at this point that these designations are intended to define populations, not species, of P-450 hemoprotein. The effect of linoleic acid hydroperoxide on the P-450 hemoprotein in microsomes from phenobarbital-treated rats was about the same as in microsomes from untreated animals, except that almost twice the concentration of hydroperoxide was required to produce a 50% loss. P-450 hemoprotein of microsomes from 3-methylcholanthrene-treated rats was even more resistant to the bleaching effect of linoleic acid hydroperoxide; a 50% loss of P-450 hemoprotein was not observed until the concentration of hydroperoxide reached 140 μM. Moreover, the loss of P-450 hemoprotein with increasing concentrations of hydroperoxide was not biphasic in these microsomes. Interpretation of the results obtained with microsomes from 3-methylcholanthrene-treated rats is complicated by the presence in these microsomes of both cytochrome P_1-450 and the cytochrome P-450 that was present before 3-methylcholanthrene was administered (17). A small, unexplained increase in P-450 hemoprotein was observed consistently in the presence of 10 or 20 μM linoleic acid hydroperoxide.

In contrast to P-450 hemoprotein, cytochrome b_5 was not readily acted upon by linoleic acid hydroperoxide; no loss of cytochrome b_5 was observed in microsomes from treated or untreated animals until concentrations of hydroperoxide exceeded 150 μM.

No apparent cytochrome P-420 was formed when linoleic acid hydroperoxide

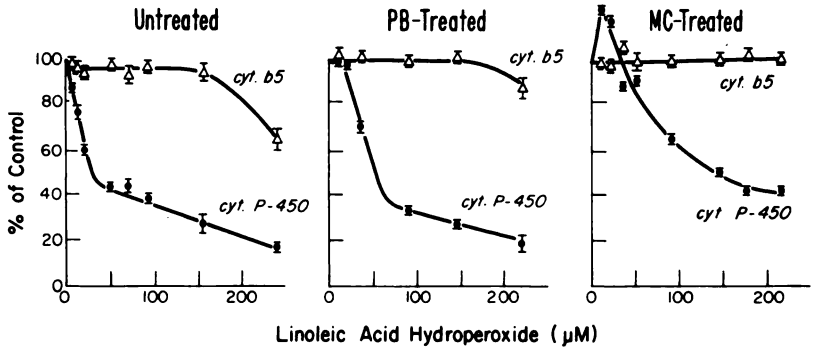

FIG. 3. *Effect of linoleic acid hydroperoxide on microsomal cytochrome levels*

Values are the means ± standard errors of at least 10 experiments. One hundred per cent values are those obtained in the absence of linoleic acid hydroperoxide (Table 1). PB, phenobarbital; MC, 3-methylcholanthrene.

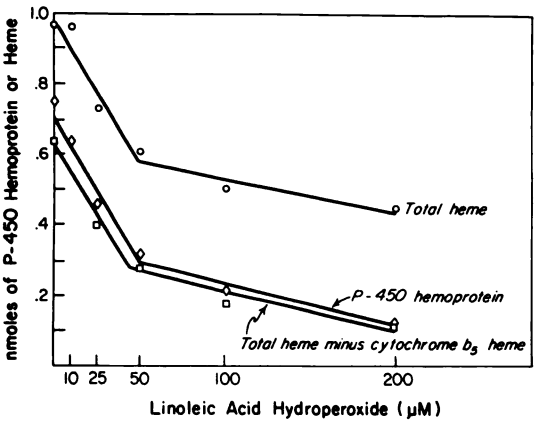

FIG. 4. *Destruction of heme of P-450 hemoprotein by linoleic acid hydroperoxide*

Hepatic microsomes were treated with increasing concentrations of linoleic acid hydroperoxide at room temperature as described in Fig. 1. After 20 min, glutathione (2.0 mM) was added, and samples were assayed for their P-450 hemoprotein (◇) and heme (○) contents. Total microsomal heme minus cytochrome b_5 heme (□) was calculated. The mean cytochrome b_5 content of untreated microsomes was determined to be 0.33 nmole/mg of microsomal protein. The results are the means of three separate experiments.

was added to microsomes. Cytochrome P-420 formed by treatment of microsomes with steapsin (13) was rapidly decolorized by linoleic acid hydroperoxide (data not shown); thus any cytochrome P-420 that might have been formed from P-450 hemoprotein through the action of linoleic acid hydroperoxide would probably have been destroyed before it could be observed by our methods.

Destruction of heme of P-450 hemoprotein by linoleic acid hydroperoxide. The destruction of the heme of P-450 hemoprotein in microsomes from untreated rats with increasing concentrations of linoleic acid hydroperoxide was determined (Fig. 4). Because cytochrome b_5 is the only hemoprotein in microsomes other than P-450 hemoprotein, and because it is not destroyed by linoleic acid hydroperoxide until very high concentrations are reached (Fig. 3), the loss of heme is due entirely to the destruction of P-450 hemoprotein. In Fig. 4 it can be seen that the loss of heme was biphasic and that it corresponded with losses of the $P\text{-}450_{(II)}$ and $P\text{-}450_{(Is)}$ phases.

Effects of linoleic acid hydroperoxide on metabolism and binding of type I compounds. The effects of linoleic acid hydroperoxide on the *N*-demethylation of ethylmorphine and the spectral binding of hexobarbital are shown in Fig. 5. Like P-450 hemoproteins, ethylmorphine *N*-demethylase activity and the hexobarbital binding spectrum[2] were destroyed biphasically when increasing concentrations of linoleic acid hydroperoxide were added to microsomes from untreated and phenobarbital-treated rats, whereas destruction of demethylation was monophasic in microsomes from 3-methylcholanthrene-treated rats. Losses of demethylase activity and

[2] Hexobarbital was used for the binding studies rather than ethylmorphine, because the type I spectrum produced by hexobarbital is induced by phenobarbital, whereas that produced by ethylmorphine is not (18).

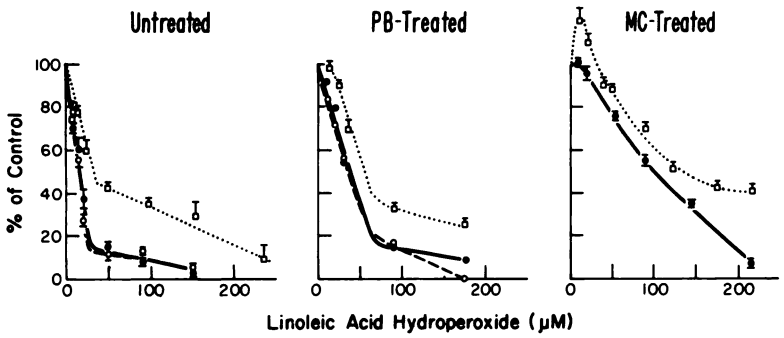

FIG. 5. *Effect of linoleic acid hydroperoxide on microsomal ethylmorphine N-demethylase activity and hexobarbital binding (type I)*

●——●, ethylmorphine *N*-demethylase activity; ○- - -○, hexobarbital binding; □· · · · □, cytochrome P-450. Values are the means ± standard errors of at least four experiments. One hundred per cent values are those obtained in the absence of linoleic acid hydroperoxide (Table 1). PB, phenobarbital; MC, 3-methylcholanthrene.

the hexobarbital binding spectrum in microsomes from untreated and phenobarbital-treated rats coincided,[3] but the losses of P-450 hemoprotein did not. Thus more than 80% of the demethylation and binding were lost when only 50–60% of the P-450 hemoprotein had been destroyed. This is interpreted to mean that $P\text{-}450_{(II)}$ is associated with high demethylase activity and type I spectral binding, and that $P\text{-}450_{(Is)}$ is associated with low demethylase activity and low type I spectral binding. These relationships were not seen when microsomes from 3-methylcholanthrene-treated rats were employed, in which case demethylase activity correlated quite well with the P-450 hemoprotein content of the microsomes at all but the highest concentrations of linoleic acid hydroperoxide.

The correlation between loss of demethylase activity and loss of P-450 hemoprotein is meaningful only if rates of demethylation were linear throughout the selected incubation periods at all levels of linoleic acid hydroperoxide addition. Microsomes from untreated rats were treated with 0, 20, and 100 μM linoleic acid hydroperoxide, which destroyed 0%, 33%, and 70% of the P-450 hemoprotein, respectively. With all three preparations, rates of metabolism of both ethylmorphine and aniline were linear throughout incubation periods of 15 and 20 min, respectively.

Effects of linoleic acid hydroperoxide on metabolism and binding of a type II compound. Aniline hydroxylase activity and the type II binding spectrum of aniline were destroyed biphasically by increasing concentrations of linoleic acid hydroperoxide in microsomes from all three sources (Fig. 6). Thus the pattern of destruction of aniline hydroxylase activity was similar to that observed when ethylmorphine was the substrate, except that destruction of demethylase activity was monophasic when microsomes from 3-methylcholanthrene-treated rats were used. Loss of type II binding correlated well with loss of hydroxylase activity when microsomes from phenobarbital- and 3-methylcholanthrene-treated rats were employed, but not when microsomes from untreated rats were used. In this case essentially all hydroxylase activity had been lost while 40% of the P-450 hemoprotein and 30% of the type II binding remained. This lack of correlation between type II binding and hydroxylation of aniline has been observed previously in microsomes from untreated rats that were not treated with linoleic acid hydroperoxide (21).

Effects of linoleic acid hydroperoxide on NADPH- and NADH-cytochrome c reductases. Irrespective of the tissue source, microsomal NADPH-cytochrome *c* reduc-

[3] The effect of linoleic acid hydroperoxide on hexobarbital binding in microsomes from 3-methylcholanthrene-treated rats is not observable; hexobarbital produces a reverse type I spectrum with these microsomes (19, 20).

tase was unaffected by linoleic acid hydroperoxide until very high concentrations of linoleic acid hydroperoxide were reached (Fig. 7). NADH-cytochrome *c* reductase activity was also unaffected by linoleic acid hydroperoxide in microsomes from untreated rats, but, in contrast to NADPH-cytochrome *c* reductase activity, it was greatly enhanced in microsomes from phenobarbital-treated rats after addition of linoleic acid hydroperoxide and was slightly enhanced in microsomes from 3-methylcholanthrene-treated animals. The increase in activity can be regarded as restoration rather than enhancement. From absolute values given in Table 1 it can be seen that NADPH-cytochrome *c* reductase was not altered greatly by phenobarbital or 3-methylcholanthrene administration; activity was increased only 15% and 4%, respectively. On the other hand, phenobarbital and 3-methylcholanthrene administration caused 40% and 20% decreases in NADH-cytochrome *c* reductases, respectively. Thus the effect of linoleic acid hydroperoxide on microsomes from the phenobarbital- and 3-methylcholanthrene-treated rats was to raise their NADH-cytochrome *c* reductase activities to the level found in microsomes from untreated rats.

Effect of linoleic acid hydroperoxide on NADPH oxidase. Figure 8 shows that NADPH oxidase was not affected by con-

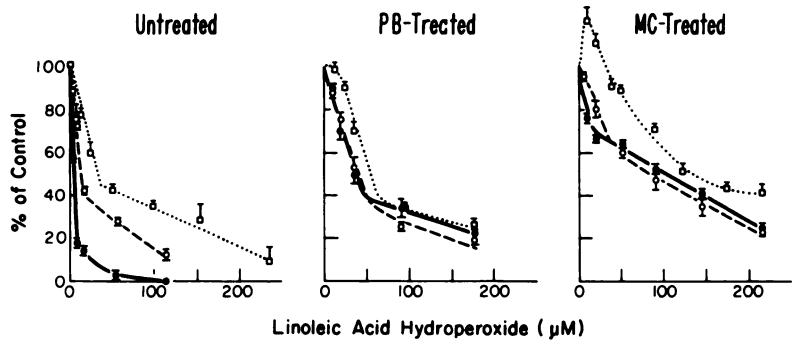

FIG. 6. *Effect of linoleic acid hydroperoxide on microsomal aniline p-hydroxylation and aniline binding (type II)*

●——●, aniline *p*-hydroxylase activity; O- - -O, aniline binding; □· · · □, cytochrome P-450. Values are the means ± standard errors of four experiments. One hundred per cent values are those obtained in the absence of linoleic acid hydroperoxide (Table 1). PB, phenobarbital; MC, 3-methylcholanthrene.

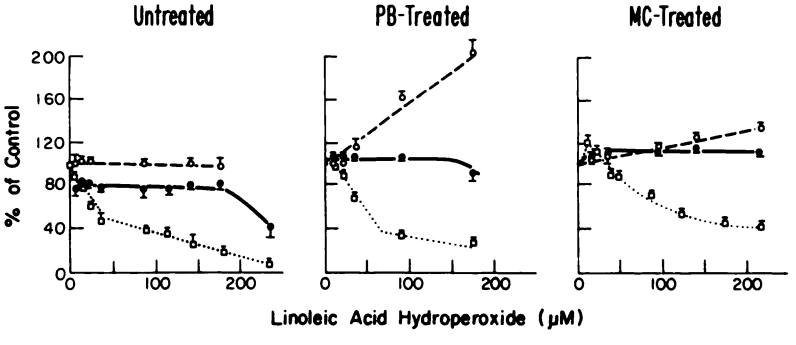

FIG. 7. *Effect of linoleic acid hydroperoxide on microsomal NADPH- and NADH-cytochrome c reductase activities*

●——●, NADPH-cytochrome *c* reductase activity; O- - -O, NADH-cytochrome *c* reductase activity; □· · · □, cytochrome P-450. Values are the means ± standard errors of four experiments. One hundred per cent values are those obtained in the absence of linoleic acid hydroperoxide (Table 1). PB, phenobarbital; MC, 3-methylcholanthrene.

TABLE 1

Components, enzyme activities, and substrate binding properties of microsomes not treated with linoleic acid hydroperoxide (100% values used in Figs 1-5)

Measurement[a]	Untreated	Phenobarbital-treated	3-Methylcholanthrene-treated
Cytochrome P-450 (nmoles)	0.56	1.02	0.87
Cytochrome b_5 (nmoles)	0.37	0.39	0.46
Ethylmorphine *N*-demethylation (nmoles/min)	4.0	23.0	5.3
Aniline hydroxylase (nmoles/min)	0.30	2.05	0.25
Hexobarbital binding ($\Delta A_{385-420}$) (ΔA/mg)	0.045	0.037	
Aniline binding ($\Delta A_{432-393}$) (ΔA/mg)	0.064	0.041	0.083
NADPH oxidation (nmoles/min)			
−Ethylmorphine (A)	7.5	21.4	14.0
+Ethylmorphine (2.0 mM) (B)	11.1	43.4	19.3
B − A	3.6	22.0	5.3
NADPH-cytochrome *c* reductase (nmoles/min)	353	405	368
NADH-cytochrome *c* reductase (nmoles/min)	1536	927	1315

[a] Values (means of at least three experiments) are in units per milligram of protein.

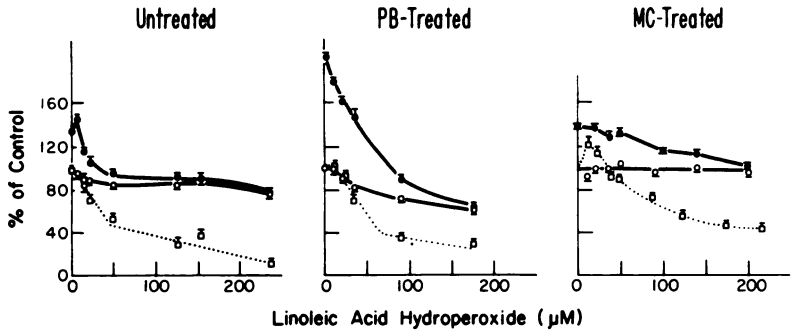

FIG. 8. *Effect of linoleic acid hydroperoxide on NADPH oxidase activity*

●——●, NADPH oxidation in the presence of 2 mM ethylmorphine; ○——○, NADPH oxidation in the absence of ethylmorphine; □· · · □, cytochrome P-450. Values are the means ± standard errors of three experiments. One hundred per cent values are those obtained in the absence of linoleic acid hydroperoxide (Table 1). PB, phenobarbital; MC, 3-methylcholanthrene.

centrations of linoleic acid hydroperoxide as high as 200 μM when microsomes from untreated and 3-methylcholanthrene-treated rats were used. When microsomes from phenobarbital-treated rats were used, a gradual loss of NADPH oxidase activity was seen with increasing concentrations of linoleic acid hydroperoxide; at a concentration of 180 μM, the loss was about 40%. With microsomes from all three sources, increases in rates of NADPH oxidation caused by addition of ethylmorphine were equal to the rates of ethylmorphine *N*-demethylation observed at each concentration of linoleic acid hydroperoxide (Fig. 5).

DISCUSSION

One of the advantages of working with soluble enzyme systems is that concentrations of individual components of the systems can be adjusted to suit the objectives of the experiment. A major disadvantage of working with membrane-bound systems

is that concentrations of individual components are established *in vivo* and usually cannot be manipulated *in vitro* without also disturbing those characteristics of the membrane which impart special properties to the bound systems. The current study describes a procedure whereby a component of the membrane-bound hepatic monooxygenase system can be removed in a stepwise manner without affecting other electron-transferring components of the system. The procedure used synthetic linoleic acid hydroperoxide to remove membrane-bound P-450 hemoproteins. As much as 75% of these cytochromes can be removed without losses of cytochrome b_5, NADPH–cytochrome *c* reductase, or NADH–cytochrome *c* reductase.

The loss of P-450 hemoprotein from microsomes with increasing concentrations of linoleic acid hydroperoxide was biphasic when microsomes from untreated or phenobarbital-treated rats were employed. The least stable population of P-450 hemoproteins was designated cytochrome P-$450_{(II)}$, and the more stable, cytochrome $P450_{(ls)}$. Almost all of the monooxygenase activity involved in the oxidation of added substrates in associated with cytochrome P-$450_{(II)}$. Type I binding (hexobarbital) is also almost exclusively associated with cytochrome P-$450_{(II)}$. The remainder of the P-450 hemoprotein [cytochrome $450_{(ls)}$] appears not to be involved in the oxidation of exogenous substrates. The view that not all of the P-450 hemoprotein of hepatic microsomes functions as a terminal oxidase in the biotransformation of xenobiotics has been expressed from time to time by several investigators; for example, Ullrich (22) estimated from spectral binding studies that only about 12% of the P-450 hemoprotein of microsomes from untreated rats functions in the hydroxylation of cyclohexane. Our studies do not show how much of the cytochrome P-$450_{(II)}$ is involved as a terminal oxidase when drug substrates are oxidized; they show only that most of this oxidase activity is associated with that fraction of the total P-450 hemoprotein designated cytochrome P-$450_{(II)}$.

Stern and Peisach (23) stated that the sixth ligand of hepatic cytochrome P-450 is probably a mercaptide ion. Green *et al.* (24) have suggested that linoleic acid hydroperoxide destroys cytochrome P-450 by reacting with the sulfur ligand. If these are correct assumptions, the question arises as to how P-$450_{(ls)}$ is prevented from reacting with linoleic acid hydroperoxide. The difference between cytochrome P-$450_{(II)}$ and cytochrome P-$450_{(ls)}$ may be that the sulfur ligand of the former is free to react with linoleic acid hydroperoxide, whereas that of the latter may be bound to a membrane component which prevents the ligand from reacting readily with lipid peroxides. Strong attachment of cytochrome P-$450_{(ls)}$ to membrane components might also account for its low drug hydroxylase activity and type I spectral binding. When rats were administered 3-methylcholanthrene, a higher proportion of the P-450 hemoprotein existed in the linoleic acid hydroperoxide-stable form than when rats were untreated or treated with phenobarbital (Fig. 3). 3-Methylcholanthrene causes the synthesis of cytochrome P_1-450 (25). From this it can be concluded that more P_1-450 than P-450 hemoprotein exists in the linoleic acid hydroperoxide-stable form. If the instability of P-450 hemoprotein is due to the reaction of the hydroperoxide with a mercaptide ion, the stability of cytochrome b_5 to linoleic acid hydroperoxide is understandable; the coordination of the iron in cytochrome b_5 is symmetrical and consists of six octahedral ligand sites occupied by imidazole or pyrole nitrogen atoms (26).

The possibility should be considered that cytochrome P-$450_{(II)}$ and P-$450_{(ls)}$ may not be different hemoproteins or classes of hemoproteins, but that they reflect differences in the proximities of the same P-450 hemoproteins to NADPH–cytochrome *c* reductase. If we assume that cytochrome P-$450_{(II)}$ represents P-450 hemoprotein that is closely associated with its reductase, and that this P-450-hemoprotein is more susceptible to degradation by linoleic acid hydroperoxide than P-450 hemoprotein that is not closely associated with the reductase (cytochrome P-$450_{(ls)}$), then a loss of mono-

oxygenase activity would be seen only when cytochrome P-450$_{(II)}$ is destroyed. This concept requires that the cytochrome P-450$_{(Is)}$ in either untreated or linoleic acid hydroperoxide-treated microsomes does not possess monooxygenase activity because it lacks proximity to its reductase. This concept would include the possibility that qualitative features of a P-450 hemoprotein, or its degree of binding to other membrane components, or both, may determine whether or not it associates intimately with NADPH–cytochrome *c* reductase. That much of the P-450 hemoprotein may not be closely associated with its reductase is suggested by the 20:1 ratio of cytochrome P-450 to NADPH–cytochrome *c* reductase in microsomes.

In microsomes from untreated and 3-methylcholanthrene-treated rats, NADPH oxidase activity remained constant when exogenous substrate was absent, regardless of the degree of depletion of cytochrome P-450 by linoleic acid hydroperoxide (Fig. 8). If all of the NADPH oxidase activity was mediated through cytochrome P-450, one would have to conclude that all or nearly all endogenous NADPH oxidation is catalyzed by cytochrome P-450$_{(Is)}$. Stoichiometric relationships show that essentially all of the increase in NADPH oxidation caused by the presence of ethylmorphine was due to its *N*-demethylation (8) (Figs. 5 and 8). Because the disappearance of ethylmorphine *N*-demethylase activity and the loss of the increased rate of oxidation of NADPH caused by the addition of ethylmorphine both coincided with the loss of cytochrome P-450$_{(II)}$ (Figs. 5 and 8), it can be concluded that when ethylmorphine is present, cytochrome P-450$_{(II)}$ functions only in the oxidation of ethylmorphine; in the presence of NADPH, cytochrome P-450$_{(II)}$ does not turn over when exogenous substrate is absent, and cytochrome P-450$_{(Is)}$ turns over at the same rate whether exogenous substrate is present or not. Cytochrome P-450$_{(II)}$ is activated only when exogenous substrate is added. In short, cytochrome P-450$_{(II)}$ would appear to function only in the oxidation of exogenous substrates, and cytochrome P-450$_{(Is)}$, only in the oxidation of endogenous substrates. This is not difficult to rationalize if cytochrome P-450$_{(Is)}$ is tightly bound to membrane components which can also serve as substrates and if cytochrome P-450$_{(II)}$ is not bound to oxidizable membrane components or is bound to oxidizable components which can be quite readily displaced by exogenous substrates.

These studies do not attempt to define the kind of endogenous NADPH oxidation that occurs when NADPH is incubated with microsomes—whether it involves the hydroxylation of phospholipid fatty acids or steroids, the production of hydrogen peroxide, the formation of superoxide, or the uncoupling of activated oxygen to form water—but merely point out that these reactions are apparently not directly affected by the presence of substrate, which appears to use only cytochrome P450$_{(II)}$, a cytochrome not involved in endogenous NADPH oxidation. The possibility should be considered that not all endogenous NADPH oxidation is mediated through P-450 hemoprotein.

Ernster and Orrenius (27) and Jansson and Schenkman (28) observed that NADH–cytochrome *c* reductase was depressed in microsomes from rats that had been treated with phenobarbital. We have confirmed this finding and shown that the addition of linoleic acid hydroperoxide restores the activity of these microsomes to the level seen in microsomes from untreated rats (Fig. 7). We have no immediate explanation for either the depressant effect of phenobarbital or the restorative action of linoleic acid hydroperoxide. Jansson and Schenkman (28) showed that NADH-dependent reductase activity is not depressed in microsomes from phenobarbital-treated rats when ferricyanide replaces cytochrome *c* as the electron acceptor in the reaction. We have confirmed this observation and shown further that linoleic acid hydroperoxide (200 μM) does not alter NADH-ferricyanide reductase activity (data not shown). In the NADH–cytochrome *c* reductase reaction, cytochrome *c* accepts electrons from NADH via cytochrome b_5 (29). Thus it would appear that

the depression of reductase activity seen after phenobarbital administration occurs at the level of cytochrome b_5.

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