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NADPH- AND LINOLEIC ACID HYDROPEROXIDE-INDUCED LIPID PEROXIDATION AND DESTRUCTION OF CYTOCHROME P-450 IN HEPATIC MICROSOMES*

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Abstract—Temporal aspects of the effects of inhibitors on hepatic cytochrome P-450 destruction and lipid peroxidation induced by NADPH and linoleic acid hydroperoxide (LAHP) were compared. In the absence of added Fe^{2+}, NADPH-induced lipid peroxidation in hepatic microsomes exhibited a slow phase followed by a fast phase. The addition of Fe^{2+} eliminated the slow phase, thus demonstrating that iron is a rate-limiting component in the reaction. EDTA, which complexes iron, and *p*-chloromercurobenzoate (pCMB), which inhibits NADPH–cytochrome P-450 reductase, inhibited both phases of the reaction. Catalase as well as scavengers of hydroxyl radical, inhibited NADPH-induced lipid peroxidation almost completely. GSH also inhibited the NADPH-dependent reaction but only when added at the beginning of the reaction. In contrast with NADPH-dependent lipid peroxidation, the autocatalytic reaction induced by LAHP was not biphasic, NADPH-dependent or iron-dependent, nor was it inhibited by hydroxyl radical scavengers, catalase or GSH. A synergistic effect on lipid peroxidation was observed when both NADPH and LAHP were added to microsomes. It is concluded that both the fast and slow phases of NADPH-dependent microsomal lipid peroxidation are catalyzed enzymatically and are dependent upon Fe^{2+}, whereas LAHP-dependent lipid peroxidation is autocatalytic. Since the fast phase of enzymatic lipid peroxidation occurred during the fast phase of destruction of cytochrome P-450, it is postulated that iron made available from cytochrome P-450 is sufficient to promote optimal lipid peroxidation. Since catalase and hydroxyl radical scavengers inhibited NADPH-dependent but not LAHP-dependent lipid peroxidation, it is concluded that the hydroxyl radical derived from H_2O_2 is the initiating active-oxygen species in the enzymatic reaction but not in the autocatalytic reaction.

Lipid peroxidation can be initiated in microsomes by the addition of NADPH [1–4] or by fatty acid hydroperoxides [5, 6]. Lipid peroxides formed during lipid peroxidation rapidly destroy hepatic microsomal cytochrome P-450 [5–9]. The current investigation examined the two peroxidative mechanisms with respect to destruction of cytochrome P-450 by observing the time courses of NADPH-induced and linoleic acid hydroperoxide (LAHP)-induced destruction of cytochrome P-450 and by using inhibitors of the reactions.

In most studies of NADPH-induced microsomal lipid peroxidation, ferrous iron or complexes of ferrous or ferric iron have been used to stimulate the reaction [10, 11]. We have observed that the reaction proceeds slowly without the addition of iron or iron complexes and that this slow phase is followed by a rapid phase. Attempts were made to characterize the fast and slow phases of the reaction.

MATERIALS AND METHODS

Preparations of microsomes. Male Sprague–Dawley rats (200–280 g), fed water and laboratory chow *ad lib*, were decapitated, and their livers were perfused with ice-cold KCl solution (1.15%). Microsomes without the glycogen fraction of the pellet were prepared by differential centrifugation as described previously [12] and washed by resuspension in 1.15% KCl and recentrifugation for 30 min at 105,000 *g*. The washed microsomes were free of hemoglobin as evaluated spectrophotometrically. All microsomal assays were carried out in 0.1 M potassium phosphate buffer, pH 7.4.

Estimation of loss of cytochrome P-450. Destruction of cytochrome P-450 was initiated by NADPH or by linoleic acid hydroperoxide (LAHP). The loss was estimated from either (a) the reduced minus reduced-CO difference spectrum of microsomes [13] or (b) by monitoring the loss of the absorption of the oxidized hemoprotein in microsomes at 418 nm [9]. Method (b) is feasible because the concentrations of LAHP used in these studies do not destroy cytochrome b_5 [8], which also has an absorption maximum at 418 nm, and because the spectrum of oxidized cytochrome P-450 is not altered with increasing additions of LAHP to microsomes [9]. Results obtained by the two methods were in excellent agreement. When method (b) was used in an assay system initiated by NADPH, the nucleotide was generated by glucose-6-phosphate dehydrogenase (0.42 units/ml), glucose-6-phosphate (4 mM), and $NADP^+$ (400 μM). NADH (200 μM) was added to the buf-

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fered microsomal suspension containing glucose-6-phosphate and $NADP^+$ to reduce cytochrome b_5, and the mixture was divided between a sample and a reference cuvette placed in an Aminco DW-2 recording spectrophotometer maintained at 37°. The reaction was initiated by adding glucose-6-phosphate dehydrogenase. When the loss of cytochrome P-450 was initiated with LAHP, microsomes contained neither NADH nor the NADPH-generating system. Cytochrome P-450 and b_5 were estimated by the procedures of Omura and Sato [13]. Lipid peroxidation was estimated by measuring malondialdehyde formation using the thiobarbituric acid procedure [14]. Protein was determined by the method of Lowry *et al.* [15].

Materials. NADPH, NADH, GSH, glucose-6-phosphate, soybean lipoxygenase, thiobarbituric acid, *p*-chloromercurobenzoate (pCMB), D-mannitol, and sodium benzoate were obtained from the Sigma Chemical Co. (St. Louis, MO). Glucose-6-phosphate dehydrogenase was purchased from Boehringer–Mannheim Biochemicals (Indianapolis, IN), 2,5-diphenylfuran from Eastman Organic Chemicals (Rochester, NY), thiourea from the Matheson Co. (Joliet, IL), and potassium phosphate from the Fisher Scientific Co. (Chicago, IL). Linoleic acid was obtained from Nuchek Prep (Elysian, MN). Linoleic acid hydroperoxide (LAHP) was prepared enzymically as described by Funk *et al.* [16] and stored as a 20% solution (w/v) in ethyl acetate under nitrogen at −70°. The purity of the hydroperoxide was verified by thin-layer chromatography [16] immediately before use by using a solvent system consisting of petroleum ether–diethyl ether–acetic acid (100:100:1).

RESULTS

Effects of Fe^{2+}, pCMB, EDTA and GSH on the time–course of NADPH-induced lipid peroxidation. Figure 1 shows that the rate of NADPH-induced lipid peroxidation in the absence of added iron began very slowly, accelerated with time, and became linear after about 10 min of incubation. Fe^{2+} (30 μM) eliminated the slow phase and increased the rate of the fast phase, thus demonstrating the rate limitation of iron. The essentiality of iron in both the induction and maintenance of NADPH-dependent lipid peroxidation is evidenced by the inhibitory effect of EDTA on both the slow and rapid phases of the reaction (Fig. 1). Our system differs from that used by several others [11, 17–20] who have studied the time–course of the reaction in that ferrous iron was used to stimulate the reaction rather than a complex of iron with ADP or pyrophosphate.

Both slow and fast phases of the reaction were inhibited by pCMB, an inhibitor of NADPH–cytochrome P-450 reductase, which maintains iron in the reduced state. These effects of Fe^{2+}, EDTA and pCMP show that the autocatalytic reaction contributes little if anything to the formation of malondialdehyde when NADPH is incubated with microsomes. These observations support the conclusion of May and McCay [17] that NADPH-dependent lipid peroxidation is entirely enzymic.

The effect of GSH depended on when it was added to the reaction mixture; it was inhibitory when added at the beginning but had no effect when added during the fast phase (Fig. 1).

Effect of the concentration of NADPH on the time–course of lipid peroxidation. Preliminary studies that

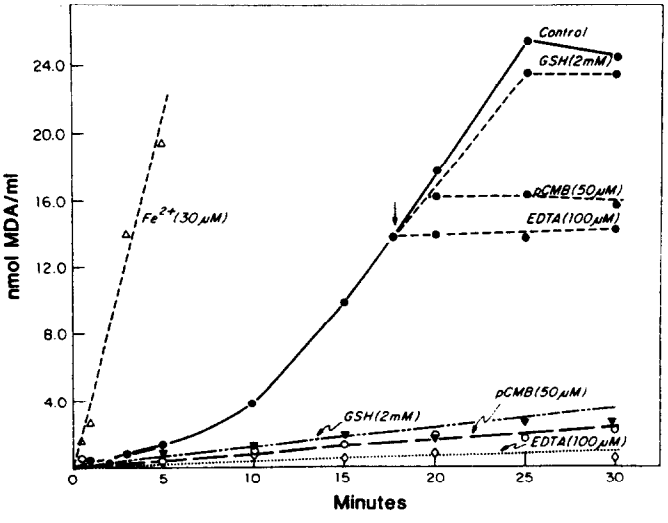

Fig. 1. Effects of Fe^{2+}, pCMB, EDTA and GSH on the time–course of NADPH-induced lipid peroxidation in microsomes. Thirty-six-milliliter volumes of microsomal suspensions (1 mg protein/ml of 0.1 M phosphate buffer, pH 7.4) were preincubated for 5 min with Fe^{2+} (ferrous ammonium sulfate) pCMB, EDTA, GSH or no addition (control). NADPH (600 μM) was then added, and duplicate 1-ml volumes of the mixtures were removed for malondialdehyde (MDA) analysis at the indicated time intervals. EDTA, pCMB or GSH was added after 18 min of incubation rather than at the beginning of the preincubation period. Each data point represents an average of a minimum of four separate experiments, each in duplicate.

employed various concentrations of NADPH showed that the fast phase of the reaction was largely non-existent at relatively low concentrations of NADPH. Figure 2 shows that the fast phase of lipid peroxidation did not occur when the concentration of NADPH was lowered from 600 to 160 μM. When the concentration of NADPH was increased to 600 μM after the microsomes had been incubated for 15 min with 160 μM NADPH, the reaction accelerated gradually to a rate equal to that observed with 600 μM NADPH. The rate of enzymatic lipid peroxidation is a function of the concentration of both iron and NADPH [14, 17]. The question was therefore raised as to whether failure of the fast phase to occur when the initial concentration of NADPH was 160 μM was due to a rate limitation of NADPH. That this was not the case was determined by monitoring the disappearance of NADPH at 340 nm when microsomes were incubated with 160 and 600 μM NADPH. The same linear rate of NADPH disappearance (5.3 nmol/mg protein/min) was seen at both concentrations during a 20-min incubation period (data not shown). The rate and time–course of lipid peroxidation was not increased with concentrations of NADPH greater than 600 μM nor when NADPH was replaced with an NADPH-generating system (400 μM $NADP^+$ + 4 mM glucose-6-phosphate + glucose-6-phosphate dehydrogenase, 0.42 units/ml) (data not shown).

Time-course of LAHP-induced lipid peroxidation. In contrast with the delayed onset of the NADPH-induced reaction (Fig. 1), LAHP-induced lipid peroxidation was almost instantaneous (Fig. 3A). When both NADPH and LAHP were added to microsomes, the amount of lipid peroxidation was greater than the sum of the lipid peroxidation induced by each agent (Fig. 3A). It is to be noted that synergism did not occur when, after 24 min of preincubation, NADPH and LAHP were combined with either LAHP or NADPH alone and that no lag phase occurred when the reaction rate of the LAHP-initiated reaction was accelerated by the addition of NADPH after 24 min of incubation (Fig. 3B).

Effects of oxygen radical scavengers on NADPH- and LAHP-induced lipid peroxidation. Singlet oxygen [21, 22], superoxide anion [10, 23], and hydroxyl radical [20, 24, 25] have been proposed as initiators of NADPH-induced lipid peroxidation. The effects of three hydroxyl radical scavengers (sodium benzoate, mannitol, thiourea) and the singlet oxygen quencher, diphenylfuran, on NADPH- and LAHP-induced lipid peroxidation were studied (Table 1). The hydroxyl radical scavengers inhibited the enzymic reaction almost completely but had no effect on LAHP-induced lipid peroxidation. Diphenylfuran had no effect on either reaction.

Effects of protein and LAHP concentration on lipid peroxidation. It can be argued that the malondialdehyde produced when LAHP is added to microsomes is derived from LAHP itself rather than from microsomal lipids. To test this possibility, the effects of protein and LAHP concentrations on malondialdehyde formation were determined. In Fig. 4 it can be seen that malondialdehyde production was a function of the protein concentration but not of the LAHP concentration. This is interpreted to mean that the malondialdehyde is derived from microsomal lipids rather than from LAHP. At the concentration of LAHP used in most of these experiments (200 μM LAHP), the contribution of LAHP *per se* to the formation of malondialdehyde, in the absence of microsomes, is negligible. Cumene hydro-

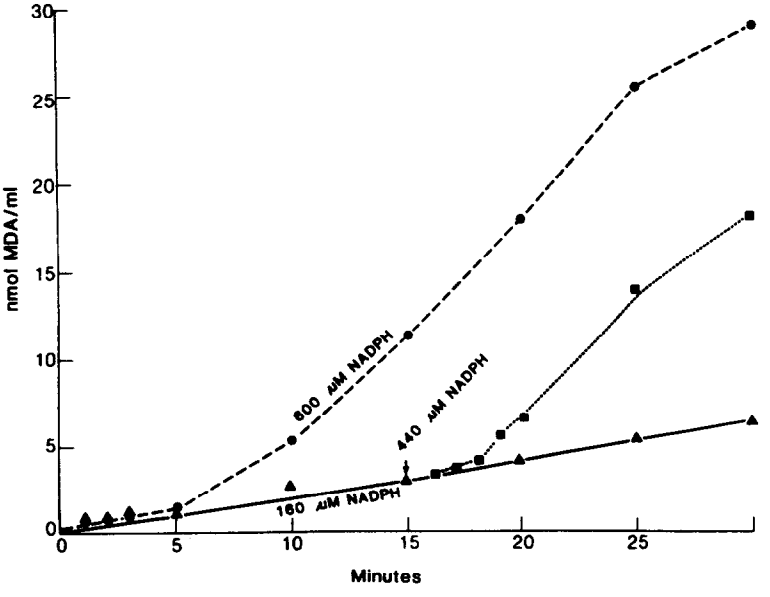

Fig. 2. Effect of the concentration of NADPH on the time–course of lipid peroxidation. Microsomal suspensions were preincubated as described in the legend of Fig. 1. Either 160 or 600 μM NADPH was added, and incubation was continued for 25 min. One-milliliter-volumes were removed for malondialdehyde (MDA) analysis at the indicated time intervals. A 440 μM concentration of NADPH was added after 15 min of incubation. Each data point represents the average of three experiments, each in duplicate.

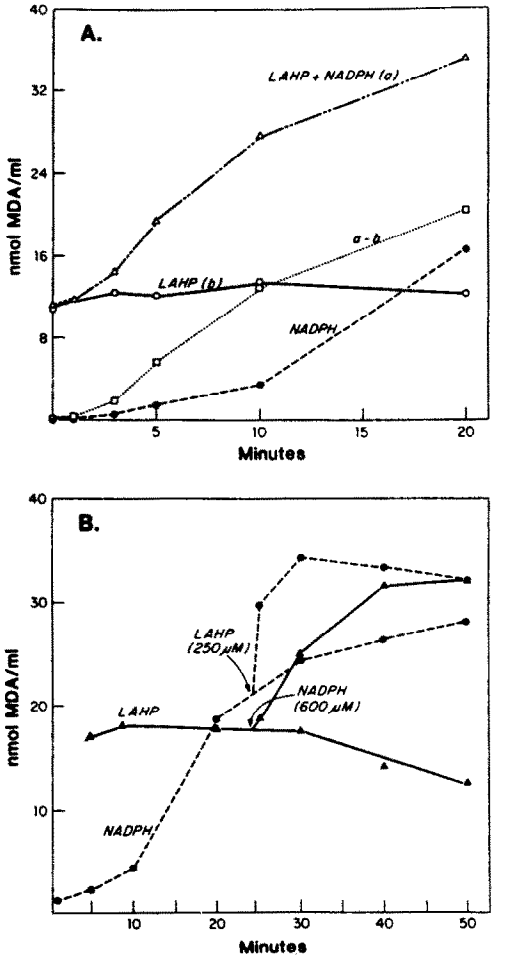

Fig. 3. Effect of LAHP on NADPH-induced lipid peroxidation and of NADPH on LAHP-induced lipid peroxidation. (A) Microsomal suspensions were preincubated as described in the legend of Fig. 1. NADPH (600 μM), LAHP (250 μM) or NADPH (600 μM) + LAHP (250 μM) was added at 0 time, and the mixtures were sampled for malondialdehyde (MDA) analysis as described in the legends of Figs. 1 and 2. (B) The experimental procedure was the same as that given in A except that NADPH and LAHP were not added together at 0 time; rather, the incubation mixture was divided after 20 min of incubation. One portion was incubated without additions and either NADPH or LAHP was added to the other portion as indicated by the arrows; both portions were incubated for another 30 min. a–b is the calculated difference of points shown on curves a and b. Each data point represents the average of three determinations.

peroxide, which is not convertible to malondialdehyde, gave results very similar to those obtained with LAHP when used in equivalent concentrations (data not shown).

Effects of EDTA, pCMB, GSH and catalase on NADPH- and LAHP-induced lipid peroxidation. Various agents that are known to alter lipid peroxidation by different mechanisms were tested for their effects on NADPH- and LAHP-induced lipid peroxidation. It can be seen in Table 2 that EDTA, pCMB, catalase and GSH inhibited NADPH-induced lipid peroxidation but not that induced by LAHP. However, with the exception of catalase, these agents did not inhibit lipid peroxidation when microsomes were incubated with both NADPH and LAHP, i.e. the rate of malondialdehyde production in each case was close to the sum of the rates of the two uninhibited reactions regardless of whether the incubation time was 3 or 20 min. Thus, it appears that LAHP reversed the inhibitory effects of EDTA,

Table 1. Effects of oxygen radical scavengers on microsomal lipid peroxidation

Addition	MDA formed (nmol/mg microsomal protein/20 min) NADPH (600 μM)	LAHP (200 μM)
None	20.8 ± 1.8	13.2 ± 1.6
Sodium benzoate (5 mM)	1.2 ± 0.3	13.0 ± 1.7
Mannitol (5 mM)	1.8 ± 0.2	14.0 ± 2.0
Thiourea (2 mM)	0.9 ± 0.2	11.2 ± 1.8
Diphenylfuran (10 mM)	19.2 ± 2.1	13.5 ± 2.0

Microsomes (1 mg protein/ml 0.1 M phosphate buffer) were preincubated at 37° with sodium benzoate, mannitol, thiourea or diphenylfuran with shaking for 3 min. Lipid peroxidation was initiated with NADPH or LAHP. The reactions were terminated after 20 min. Values are mean ± SEM for three or four experiments. MDA = malondialdehyde.

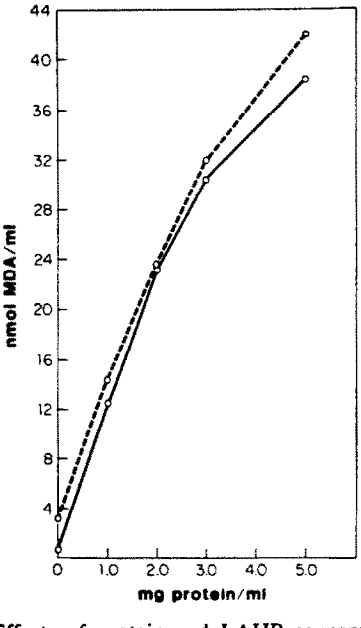

Fig. 4. Effects of protein and LAHP concentration on LAHP-induced lipid peroxidation. Lipid peroxidation in a 1-ml microsomal protein suspension in 0.1 M potassium phosphate buffer, pH 7.4, was initiated with 250 μM (O—O) or 500 μM (O- -O) LAHP. The malondialdehyde (MDA) content of this mixture was determined after 20 min of incubation at room temperature. Each data point represents the average of at least three separate determinations.

Table 2. Effects of inhibitors on NADPH- or NADPH-plus LAHP-dependent lipid peroxidation

	MDA (nmoles)					
	NADPH		LAHP		NADPH + LAHP	
Addition	3 min	20 min	3 min	20 min	3 min	20 min
None	0.4	20.1 ± 1.5	21.9	18.0 ± 1.6	23.7	33.0 ± 2.8
EDTA (50 μM)	0.3	0.5 ± 0.1	21.3	16.8 ± 1.7	23.0	35.0 ± 3.1
pCMB (50 μM)	0.2	1.6 ± 0.3	23.0	16.6 ± 2.0	25.6	30.4 ± 3.6
Catalase (20 μg)		2.0 ± 0.5		17.0 ± 2.0		15.5 ± 2.0
GSH (2 mM)	0.6	4.2 ± 0.8	20.0	15.3 ± 1.0	24.5	36.3 ± 3.3

Microsomes (1 mg protein/ml 0.1 M phosphate buffer) were preincubated at 37° with EDTA, pCMB, catalase (1 μg = 20 units) or GSH, with shaking for 3 min. Lipid peroxidation was initiated with NADPH (600 μM), LAHP (250 μM) or both. Reactions were terminated after 3 or 20 min. Values are nmol malondialdehyde (MDA) formed in 20 min (mean ± SEM, N = 3) or 3 min (means of two experiments).

pCMB and GSH but not that of catalase, on the NADPH-supported reaction.

Relation of loss of cytochrome P-450 to NADPH- and LAHP-induced lipid peroxidation. Loss of microsomal P-450 has been associated with both NADPH- [1] and LAHP- [5–10] induced lipid peroxidation. Differences in the time–courses of lipid peroxidation produced by NADPH and LAHP afford an opportunity to differentiate the two mechanisms of lipid peroxidation with respect to the destruction of cytochrome P-450. Figure 5 shows that the loss of cytochrome P-450 was very rapid when LAHP was used but, when NADPH was used, only a very slow loss of the hemoprotein occurred during an initial lag period of about 6 min. These temporal aspects of the losses of cytochrome P-450 correspond to the rapid and slow induction of lipid peroxidation by LAHP and NADPH respectively (Figs. 1 and 3). Figure 5 also shows that NADPH reinitiated LAHP-induced loss of cytochrome P-450 after it had plateaued; conversely, after NADPH-induced loss of cytochrome P-450 had plateaued, it was reinitiated with LAHP. In summary, these studies demonstrate excellent correlation of loss of cytochrome P-450 with generation of lipid peroxidation regardless of whether either effect was initiated by NADPH or by LAHP.

Figure 6 shows the effects of EDTA and pCMB on NADPH- and LAHP-induced losses of cytochrome P-450. As was anticipated from the effects of these agents on lipid peroxidation (Table 2), both inhibitors prevented NADPH-induced loss of cyto-

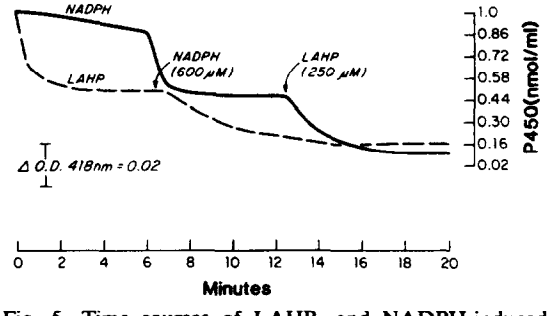

Fig. 5. Time–courses of LAHP- and NADPH-induced losses of microsomal cytochrome P-450. A 5-ml microsomal suspension (1 mg protein/ml in 0.1 M potassium phosphate buffer, pH 7.4, containing 150 μM NADH) was divided equally between two cuvettes and placed in the sample and reference cuvette chambers of an Aminco DW-2 spectrophotometer maintained at 37°. Destruction of cytochrome P-450 was initiated by plunging either NADPH (1.5 μmol) or LAHP (0.63 μmol) into the sample cuvette. The loss of cytochrome P-450 was recorded at 418 nM in the split beam mode. Additional LAHP or NADPH was added to the sample cuvette at the time intervals indicated by arrows. The experiments were performed at least four times with consistent results.

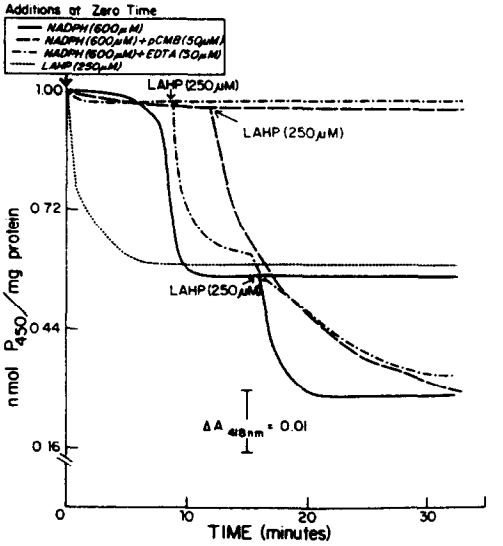

Fig. 6. Effects of EDTA and pCMB on NADPH- and LAHP-induced losses of microsomal cytochrome P-450. Losses of cytochrome P-450 were monitored as described in the legend of Fig. 5. The mixtures were divided at the time indicated by arrows; one portion was incubated with and the other without LAHP. The experiments were performed at least three times with consistent results.

chrome P-450 but were without effect on the LAHP-induced loss of cytochrome P-450. Also in accordance with the observed lack of effects of EDTA and pCMB on lipid peroxidation when both NADPH and LAHP were present (Table 2), NADPH-induced loss of cytochrome P-450 was not prevented by either inhibitor in microsomes that had been pretreated with LAHP. Thus, in the presence of EDTA or pCMB as much cytochrome P-450 was lost when either of these inhibitors was present as when either was absent. When EDTA or pCMB was present in the system containing LAHP and NADPH, the rapid rate of destruction caused by LAHP was followed by an additional slow loss of cytochrome P-450 which continued until the total loss equaled the sum of the individual losses of cytochrome P-450 produced by LAHP and NADPH in the absence of these inhibitors. However, the additional loss of cytochrome P-450 due to NADPH above that caused by LAHP proceeded at a rate that was only about 1/100 of the rapid reaction that was seen when NADPH alone was added to microsomes. The sudden break in the curve seen about 5 min after the addition of LAHP to the medium containing NADPH and EDTA was a consistent finding; it was not observed when pCMB was substituted for EDTA.

DISCUSSION

Two general mechanisms for microsomal lipid peroxidation have been described; one is initiated enzymically, the other occurs spontaneously when lipid peroxides are present. Singlet oxygen [21, 22], superoxide anion [10, 24, 225], and the hydroxyl radical [22, 24, 25] have been implicated in the enzymic reaction. In our system, singlet oxygen is not a likely candidate for the initiating active oxygen species because diphenylfuran, a singlet oxygen scavenger [24], did not inhibit microsomal MDA formation (Table 1). Hydroxyl radical is the likely active form of oxygen because MDA formation was inhibited almost completely by the hydroxyl free radical trapping agents, sodium benzoate, mannitol and thiourea [26, 27] (Table 1). Our observed inhibition of lipid peroxidation by hydroxyl radical scavengers agrees with the findings of some investigators [22, 25, 26, 28] but not with those of others [10, 29]. Also discordant with our findings is the resport of Morehouse and associates [30] of the non-involvement of either H_2O_2 or OH· in lipid peroxidation. It should be noted that the lack of effect of hydroxyl radical scavengers seems to be reported mostly in systems in which chelators (e.g. EDTA, ADP) are added with iron. In our system, we did not add exogenous chelators, although the phosphate ion in our buffer is a good iron chelator [31] that may have contributed, as may unidentified chelators present in the microsomes. In our opinion, the controversy regarding the involvement of the hydroxyl radical in lipid peroxidation reflects the multiplicity of mechanisms of oxygen activation in lipid peroxidation, any of which may predominate depending on the composition of the reaction medium. Superoxide is not the active form of oxygen because benzoate, mannitol and thiourea do not scavenge superoxide. Hydroxyl radical is believed to be formed through the iron-catalyzed Haber–Weiss and Fenton reactions as follows [25, 32].

$$Fe^{2+} + O_2 \longrightarrow O^{2-} + Fe^{3+} \quad (1)$$

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^\cdot + OH^- + Fe^{3+} \quad (3)$$

The inhibition of the reaction by catalase (Table 2) adds credence to the view that enzymic lipid peroxidation is initiated by the hydroxyl radical derived from hydrogen peroxide [20, 28] (reaction 3).

Non-enzymic catalysis of linoleic acid hydroperoxide (LOOH) is believed to proceed autocatalytically and to involve the following reactions [33–35]:

$$LOOH + Fe^{3+} \ldots\ldots LOO^\cdot + Fe^{2+} + H^+ \quad (4)$$

$$LOOH + Fe^{2+} \ldots\ldots LO^\cdot + OH^- + Fe^{3+} \quad (5)$$

$$2\,LOOH \ldots\ldots LOO^\cdot + LO^\cdot + H_2O \quad (6)$$
$$(4 + 5)$$

Catalase did not inhibit LAHP-induced MDA formation (Table 2) because hydrogen peroxide is not involved. The non-involvement of the hydroxyl radical in these reactions would explain the failure of sodium benzoate, mannitol and thiourea to inhibit MDA formation (Table 1). The observed differences in the inhibitory effects of these agents on NADPH- and LAHP-induced lipid peroxidation reinforces the view that different mechanisms are involved in the two reactions.

In the absence of added iron, the enzymic reaction proceeded in two phases, a slow phase and a fast phase (Fig. 1). Although this temporal profile fits that of an autocatalytic reaction [33–35], both phases of the reaction were enzymic. This conclusion is based largely on three observations: (a) pCMB, which inhibits NADPH–cytochrome P-450 reductase [17], blocked the fast phase of the NADPH-induced reaction completely (Fig. 1); (b) a fast phase did not develop when a low concentration of NADPH was used to promote a prolonged lag phase, as it should have if lipid hydroperoxides had been accumulating during this lag phase and were autocatalytically peroxidative. Moreover, a fast phase occurred when more NADPH was added after the prolonged slow phase (Fig. 1); and (c) although the EDTA–iron complex may stimulate enzymic lipid peroxidation under certain conditions [18, 21], as, for example, when reconstituted systems and Tris buffer are used, EDTA inhibits the reaction in the concentration and under the conditions employed in our studies and in those of many other investigators [36–39]. The iron for the slow phase is supplied by undetermined amounts of free or dissociable iron contained in the microsomes. Free ferric iron does not catalyze microsomal lipid peroxidation [14]; thus, for this microsomal iron to act catalytically, it would have to be reduced to the ferrous form or, if it were in the ferric form, by complexing with microsomal components in a way that would allow it to react with the reductase. Only a very small amount of this iron would be required to catalyze the slow phase of this reaction. The K_m of iron for microsomal lipid peroxidase is 1 μM [14, 40]. This concentration

approximates that present in our buffer, yet a lag phase of lipid peroxidation was observed. However, the iron in the buffer is ferric iron, which did not support lipid peroxidation in our experiments. Microsomal lipid peroxidation involves several steps, including (a) oxygen uptake and activation, (b) oxygenation of unsaturated membrane lipids to yield hydroperoxide derivatives, (c) cleavage of the unsaturated lipid hydroperoxide, and (d) decomposition of the hydroperoxide into other products, including MDA. Our observations suggest that each of these reactions may be enzymic, with requirements for critical concentrations of iron and NADPH. Thus, the inability of 160 μM NADPH to support maximum rates of lipid peroxidation in our system may be due to a high K_m of NADPH for any or all of the above reaction steps, particularly when concentrations of iron are suboptimal. Indeed, it has been shown that the cleavage reaction (reaction c above) has a very high K_m of iron and may be rate-limiting for MDA production [4]. Based on our findings, and as suggested previously [40], it is unlikely that autocatalysis contributes significantly to any of the above four reaction steps during NADPH-dependent microsomal lipid peroxidation. Since the fast phase is predominantly enzymic, it follows that the rate limitation of iron is overcome as the reaction proceeds from the slow to the fast phase. The iron for the fast phase of lipid peroxidation may be derived from cytochrome P-450 in accordance with the model provided by Brown *et al.* [41] in which iron is released from ferrihemes by H_2O_2. Being lipid soluble, lipid hydroperoxides would be expected to react even more readily than H_2O_2 with cytochrome P-450 to produce iron.

Preliminary attempts to determine the iron that might have been released from cytochrome P-450 by NADPH- or LAHP-induced lipid peroxidation were abandoned when it became obvious that assays could not yield interpretable results. The lower limit of determination of free iron in biological materials by a standard procedure [42] is 2 nmol. Our microsomal preparations contained about 1 nmol of cytochrome P-450/ml; thus, with about half of the cytochrome P-450 destroyed by either NADPH or LAHP, a maximum of only 0.5 nmol of iron could be derived from cytochrome P-450 if all of this iron was released in free form. If one assumes that this cytochrome P-450-derived iron is the major catalytic iron species, the theoretical effective iron concentration would be 0.5 μM, about half the K_m of the cation for lipid peroxidation [14, 40]. NADPH–cytochrome P-450 reductase. cytochrome P-450, and phospholipid are believed to be closely associated in the microsomes [43]; thus, iron released from cytochrome P-450 would be localized at the catalytic site at a concentration exceeding that in the general medium and high enough to catalyze lipid peroxidation. Conceivably, this iron would result in a much higher local concentration of iron than could be achieved with the same amount of iron distributed throughout the medium. If this is the case, only a very small, undetectable amount of iron derived from cytochrome P-450 would be needed to catalyze the reaction. It is also possible, if not likely, that very little free iron exists in either untreated or LAHP-treated microsomes, but that the catalytic iron is bound in equilibrium between the reductase and other microsomal proteins. Meaningful analysis of iron is further complicated because microsomes take up iron from the medium. When 87 nmol of $FeCl_3$ was added to a suspension of 5 mg of microsomal protein/ml phosphate buffer, a time-dependent increase in absorbance around 425 nm was observed; the nature of the spectral complex formed with ferric hydroxide, the form in which Fe^{3+} exists in aqueous media [40], is unknown. Less than 10% of the added iron was recovered in the supernatant fraction obtained after centrifugation at 100,000 *g* for 30 min. Thirty nanomoles of added $FeCl_3$ elicited no stimulation of lipid peroxidation (data not shown). Thus, exogenous iron may be taken up by microsomes, may not be assayed as free iron, and may not catalyze lipid peroxidation. This reinforces our argument that the buffer-derived 0.92 μM Fe^{3+} does not participate in lipid peroxidation in our system.

Evidence was presented to show that NADPH- and LAHP-induced lipid peroxidations proceed independently. The NADPH-induced reaction was characterized by a slow phase followed by a fast phase. When both NADPH and LAHP were incubated with microsomes, the early time course of lipid peroxidation reflected the sum of the time–courses of the individual reactions except that the slow phase of the NADPH-induced reaction appeared to have been largely eliminated (Fig. 3). As the reaction proceeded, the rate of peroxidation exceeded the sum of the two reactions. As discussed previously, this could occur if the NADPH reaction was stimulated by iron made available by the LAHP-induced destruction of cytochrome P-450. By the end of 20 min of incubation, the synergistic effect of the LAHP-induced reaction on the NADPH-induced reaction had been almost eliminated. Synergism was not seen when LAHP was added during the fast phase of the NADPH-induced reaction (Fig. 3B). When NADPH was added to microsomes that had been incubated for 24 min with LAHP, only the rapid phase of NADPH-induced lipid peroxidation was observed (Fig. 3B). This would be expected if iron had been made available to the enzymic reaction through the destruction of cytochrome P-450 by LAHP.

EDTA and pCMB inhibited NADPH-induced lipid peroxidation. Neither of these agents inhibited LAHP-induced lipid peroxidation (Table 2). However, no apparent inhibition of lipid peroxidation by these agents was seen when both NADPH and LAHP were added to microsomes. The failure of pCMB to inhibit lipid peroxidation under these conditions could mean that LAHP had reversed the inhibitory effect of pCMB on the NADPH-induced reactions or that pCMB stimulates the rapid induction of lipid peroxidation by LAHP, which proved not to be the case (Table 2) or that some pCMB-insensitive or activated factor is involved. It is conceivable that the hypothetical pCMB-insensitive or activated factor may not contribute to lipid peroxidation in untreated microsomes but may do so after the membrane has been disrupted by LAHP. The possibility should also be considered that the treatment of microsomes with LAHP pro-

duces conditions favorable for the stimulation of lipid peroxidation by EDTA in combination with iron to yield an EDTA–iron complex, which is stimulatory to lipid peroxidation [24], and that the reduced state of this complex is maintained by the pCMB factor. Still another possibility to be considered is that, in the presence of reducing equivalents, EDTA may act as a free radical scavenger [44]; under certain conditions, radical scavengers can stimulate lipid peroxidation [34, 45]. These special peroxidation-promoting conditions may arise when the membrane is disrupted by LAHP. It should be noted that the potent antioxidant ascorbic acid also stimulates microsomal lipid peroxidation [3], perhaps by creating similar special conditions in microsomes.

The concentration of LAHP used in the studies represented by Fig. 3 was in excess of that required for maximal peroxidative activity (Fig. 4), yet it produced only about 50% of the lipid peroxidation that was achievable when both LAHP and NADPH were present (Table 2). This could mean either that for unknown reasons the autocatalytic reaction was terminated before all of the available unsaturated fatty acids had been used, or that NADPH-induced lipid peroxidation and LAHP-induced lipid peroxidation involve different pools of unsaturated fatty acids.

GSH inhibited NADPH-dependent lipid peroxidation when added at the beginning of the reaction (Fig. 1, Table 2), but not when added during the rapid phase of the reaction (Fig. 1). This time-dependent antioxidant effect of GSH can be rationalized on the basis of the initial presence of some labile GSH-dependent or microsomal factor(s) e.g. GSH-transferases [46], and the factors described by Burk [47, 48] or cytochrome P-450, which can also function as a peroxidase [6]; a subsequent loss of these protective factors might occur as the reaction proceeds. Moreover, GSH has been reported to promote lipid peroxidation under certain conditions [49]. Based on these speculations, the absence of any protection by GSH against any of the effects of LAHP can be attributed to instantaneous destruction of the GSH–dependent factor(s) by LAHP.

NADPH- and LAHP-induced losses of cytochrome P-450 correlated very well with NADPH- and LAHP-induced lipid peroxidation: (a) both cytochrome P-450 destruction and lipid peroxidation appeared to be induced independently by concentrations of NADPH and LAHP which elicited maximal lipid peroxidation; (b) EDTA and pCMB prevented NADPH-induced lipid peroxidation and destruction of cytochrome P-450 in the absence, but not in the presence, of LAHP; and (c) losses of cytochrome P-450 and lipid peroxidation were temporally correlative.

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