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Use of Linoleic Acid Hydroperoxide in the Determination of Absolute Spectra of Membrane-Bound Cytochrome P-450

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SUMMARY

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Linoleic acid hydroperoxide (LAHP) destroys cytochrome P-450 in microsomes without destroying cytochrome b_5. This is the basis for a simple and direct method of determining the absolute spectra of membrane-bound cytochrome P-450. The method consists of adding LAHP to the reference cuvette of a spectrally balanced pair of cuvettes containing the same microsomal suspension and observing the resulting difference spectrum. The absolute spectra of oxidized, reduced, or reduced carbon monoxide-complexed cytochrome P-450 in microsomes from untreated or phenobarbital- or 3-methylcholanthrene-treated rats were very similar to corresponding spectra that have been reported for a highly purified preparation of P-450LM$_2$ with the exception that the oxidized spectrum of membrane-bound cytochrome P-450 represented a mixture of high- and low-spin forms of cytochrome P-450, whereas P-450LM$_2$ gives a typical low-spin spectrum.

INTRODUCTION

The absolute spectra of hepatic membrane-bound cytochrome P-450 cannot be determined by direct spectrophotometric methods because of interference by microsomal cytochrome b_5. Kinoshita and Horie (1) circumvented this problem by recording the difference spectrum of microsomes from PB[2]-induced rabbits versus microsomes from untreated rabbits after adjusting the concentrations of microsomes in the reference and sample cuvettes so that each contained the same concentration of cytochrome b_5. Their method is based on the knowledge that cytochrome b_5 is the only interfering chromophore in hepatic microsomes and that administered phenobarbital causes a large increase in cytochrome P-450 without a corresponding increase in cytochrome b_5. The method assumes that the absolute spectra of the cytochrome P-450 induced by phenobarbital are identical with those of the P-450 hemoprotein present in microsomes from untreated animals.

We reported that LAHP can be used to bleach P-450 hemoproteins in hepatic microsomes without destroying cytochrome b_5 and other known electron transfer components (2). The addition of LAHP to the reference cuvette of a spectrally balanced pair of cuvettes containing the same microsomal suspension provides a simple, direct means of determining the absolute spectra of membrane-bound cytochrome P-450 in hepatic microsomes. We have used this procedure (LAHP deletion procedure) to determine the absolute spectra of membrane bound P-450 in microsomes from untreated, PB-treated, and MC-treated rats. The absolute Type I (hexobarbital), Type II (aniline), reverse Type I (phenacetin), and ethylisocyanide-binding spectra of microsomes were also determined.

METHODS

Animals. Male Holtzman strain rats (180–220 g) received i.p. injection of 0.9% NaCl, sodium PB (40 mg/kg/day), or MC (20 mg/kg/day) for 4 days and were killed 24 hr after the last injection.

Liver preparations. Microsomes, prepared as described previously (3), were passed through a Sepharose 2B column as described by Tangen *et al.* (4) to remove hemoglobin and nonmicrosomal protein. The eluted microsomes were diluted to 5 mg of protein/ml with phosphate buffer (pH 7.4), treated for 2 hr at 4° with various concentrations of standardized (2, 5) LAHP, diluted with phosphate buffer to 2 mg of protein/ml, and placed in a suction flask. The flask was evacuated and refilled with

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[2] The abbreviations used are: PB, phenobarbital; LAHP, linoleic acid hydroperoxide; MC, 3-methylcholanthrene.

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nitrogen twice to remove carbon monoxide (6, 7). This is necessary because carbon monoxide in the reference cuvette produces a reduced cytochrome P-450·CO spectrum which superimposes a trough at 450 nm on the reduced spectrum of the cytochrome P-450 in the sample cuvette. Control microsomes were treated in the same manner except that LAHP was omitted.

Spectra. LAHP-treated microsomes were placed in the reference cuvette and untreated microsomes in the sample cuvette. The absolute reduced cytochrome P-450 spectrum was recorded after adding a small amount of solid dithionite to both cuvettes. The absolute reduced cytochrome P-450·CO spectrum was recorded after saturating the contents of both cuvettes with CO and then adding dithionite to both cuvettes. The absolute oxidized cytochrome P-450 spectrum was recorded without adding anything to the cuvettes. Absolute Type I (8, 9), Type II (8, 9), and reverse Type I (10) spectra were recorded after adding hexobarbital (0.25 or 0.83 mM), aniline (0.10 or 0.33 mM), or phenacetin (0.33, 1.33, or 2.67 mM), respectively, to both cuvettes. Baselines for all of these recordings were established by balancing cuvettes which contained untreated, oxidized microsomes from the same liver preparation used to prepare the LAHP-treated microsomes.

Difference ethylisocyanide spectra of reduced untreated and LAHP-treated microsomes were determined as described previously (11). The P-450 hemoprotein contents of treated and untreated microsomes were determined by the method of Omura and Sato (12). We have observed that the reduced CO difference spectrum does not develop fully immediately after the addition of CO and that the time required for full development of the spectrum varies greatly from one microsomal preparation to another. Accordingly, the spectra of each microsomal preparation were scanned repeatedly until no further change in the maximal absorption at about 450 nm was observed. Some preparations required as long as 15 min for full development of the spectra; spectra obtained with MC-treated microsomes were particularly slow in developing. All spectral measurements were made with an Aminco DW-2 spectrophotometer.

Extinction coefficients. The net concentration of cytochrome P-450 responsible for a given spectrum was determined by subtracting the concentration of cytochrome P-450 in the reference cuvette (LAHP-treated microsomes), as determined by the method of Omura and Sato (12), from the concentration of cytochrome P-450 (untreated microsomes) contained in the sample cuvette, also determined by the method of Omura and Sato. Extinction coefficients were calculated from the net concentration of cytochrome P-450 using 91 mM^{-1} cm^{-1} as the extinction coefficient for cytochrome P-450 (12). Since the absolute spectra obtained by the LAHP deletion procedure employs difference spectroscopy, the baseline at zero A is not a spectral constant. Molar extinction coefficients are therefore reported as $A_{\lambda max} - A_{490\ nm}$. A small amount of cytochrome P-420 is formed when the absolute reduced CO spectrum is determined. The procedure of Nishibayashi and Sato (13) was used to correct for this source of error. A correction for cytochrome P-420 formation was usually not necessary when the reduced CO difference spectrum was determined by the conventional method (12); cytochrome P-420 is formed only when the microsomes are permitted to stand for a prolonged period of time after the addition of dithionite.

Chemicals. Sodium hexobarbital was obtained from Sigma Chemical Company, St. Louis, Mo., and phenacetin from Matheson, Coleman and Bell, Norwood, Ohio. The sources of other chemicals have been given previously (3).

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

RESULTS

Absolute spectra of oxidized, reduced, and reduced-carbon monoxide membrane-bound cytochrome P-450 from untreated, PB-treated, and MC-treated rats. The absolute spectra of oxidized, reduced, and reduced-CO membrane-bound cytochrome P-450 obtained by the LAHP deletion procedure are shown in Fig. 1. Table 1 lists the absorption maxima and extinction coefficients of the absolute spectra of membrane-bound cytochrome P-450 from untreated, PB-treated, and MC-treated rats.

Hepatic microsomes are known to contain several molecular species of cytochrome P-450 (15–18). The possibility was considered that the extinction coefficients of individual species of cytochrome P-450 might not be identical. Should this be the case, and should certain species be more susceptible to destruction by LAHP than others, the observed extinction coefficient would vary with the extent of deletion of cytochrome P-450 from the microsomes by LAHP. This possibility was tested by monitoring the maximal absorbance (417 nm) of oxidized microsomes from untreated, PB-treated, and MC-treated rats after successive additions of LAHP. A correlation between the maximal absorbance of oxidized cytochrome P-450 is meaningful only if the loss of cytochrome P-450 equals the loss of heme; this was the case (2). The extinction of the maximal absorbance of oxidized cytochrome P-450 did not change with LAHP-induced changes in the concentration of cytochrome P-450 regardless of the source of microsomes, whether from untreated, PB-treated, or MC-treated rats (Fig. 2).

Absolute spectra of cytochrome P-450 complexes with Type I, Type II, and reverse Type I compounds. Figure 3 shows the absolute spectra obtained by the LAHP deletion procedure when hexobarbital (Type I), aniline (Type II), or phenacetin (reverse Type I) was added to hepatic microsomes from untreated rats. Calculated difference spectra (oxidized microsomes plus hexobarbital, aniline, or phenacetin minus oxidized microsomes treated with LAHP) are also given in Fig. 3. The absolute Type I and Type II binding spectra are very similar to those reported by Remmer *et al.* (19) and Schenkman *et al.* (20), who used the method of Kinoshita and Horie (1). Slight differences might be expected, because in their studies the binding substance was not added to the reference cuvette, whereas in the current studies the binding substance was added to both cuvettes. However, it is not to be assumed that all of the cytochrome P-450

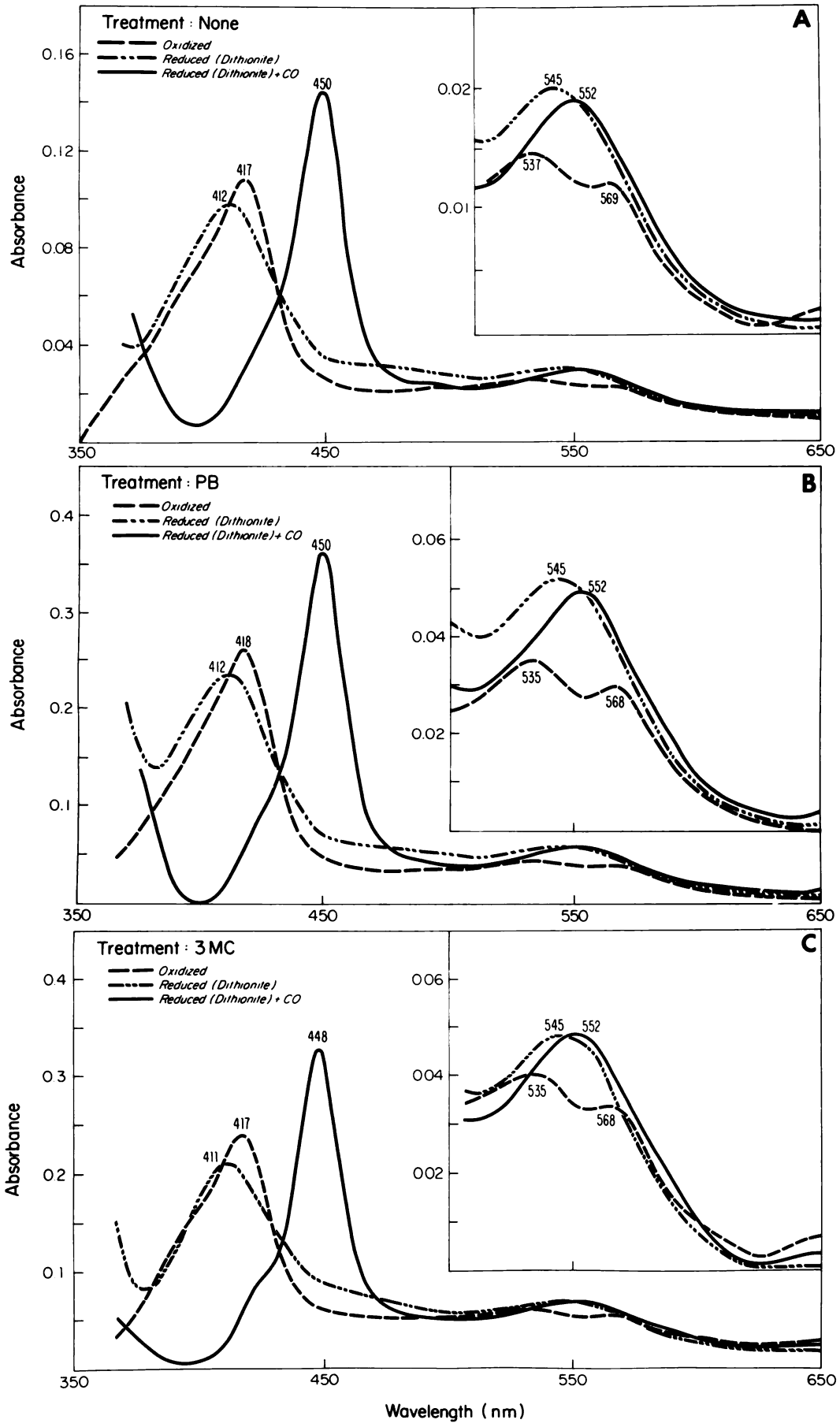

TABLE 1

Absorption maxima and molar extinction coefficients ($A_{\lambda max}$–$A_{490\ nm}$) of the absolute spectra of membrane-bound cytochrome P-450 from untreated, PB-treated, and MC-treated rats

Values for membrane-bound cytochrome P-450 were obtained using the LAHP deletion procedure described under Methods. Values are the means of three (untreated and PB-treated microsomes) or four (MC-treated microsomes) using different microsomal preparations.

Condition	Membrane source					
	Untreated microsomes		PB-treated microsomes		MC-treated microsomes	
	λmax	ε	λmax	ε	λmax	ε
Oxidized	418	60.3	417	65.4	417	65.8
	535	7.8	535	9.7	535	10.8
	570	7.8	570	8.4	570	8.7
Reduced	413	67.1	411	65.9	412	67.7
	550	15.7	550	14.2	550	14
Reduced + CO	449.6	94.5	449	98.6	447.6	108.1
	555	12.3	555	13.9	555	13.9

in either cuvette was bound to the substrate or that the same percentage of binding of substrate to the cytochrome P-450 occurred in each cuvette.

Ethylisocyanide difference spectra of untreated and LAHP-treated microsomes. The ethylisocyanide binding spectrum is characterized by two maxima, one at 430 nm and the other at 455 nm (21). The ratio of the peak heights of these maxima varies with the pH. Differences in these ratios at one or more pH unit has been used to demonstrate qualitative differences in certain P-450 hemoproteins (11). Eight successive additions of LAHP were used to destroy the cytochrome P-450 of control microsomes in approximately equal increments until about 80% had disappeared. Ethylisocyanide difference spectra of these LAHP-treated microsomes were determined at pH 6.5 and 8.5 in the usual manner (11), i.e., with LAHP-treated microsomes in both cuvettes, and ethylisocyanide added to the sample cuvette only. The 455 nm/430 nm ratio at pH 6.5 (0.39 ± 0.00 SE) and at pH 8.5 (1.11 ± 0.02 SE) did not change with progressive losses of cytochrome P-450. This indicates that individual species of the cytochrome P-450 population in untreated microsomes form very similar spectral complexes with ethylisocyanide or, if they do not, that those species of cytochrome P-450 that are destroyed by LAHP are not destroyed selectively.

DISCUSSION

The LAHP deletion procedure was used in these studies to obtain the absolute oxidized, reduced, and reduced-CO spectra of membrane-bound cytochrome P-450 in microsomes from untreated, PB-treated, and MC-treated rats. The reduced and reduced-CO spectra were very similar to those obtained with the highly purified rabbit cytochrome P-450LM$_2$ (15). In the current study, molar extinction coefficients were reported as A_{max}–$A_{490\ nm}$. If one assumes that the false zero baseline obtained by the LAHP deletion procedure deviates uniformly from the true base line at all wavelengths of the recorded spectrum, an estimate of the extinction coefficient based on the true zero baseline can be made from reported spectra of highly purified preparations of cytochrome P-450. Thus, if the values given in Table 1 for the extinctions of the Soret maxima of reduced and reduced + CO/PB-treated microsomes (66 and 99, respectively) are multiplied by the A_{max} + A_{max}–$A_{490\ nm}$ shown for the reduced and reduced-CO spectra of P-450LM$_2$ in figure 6 of a publication by Haugen and Coon (15), values of 76 and 106, respectively, are obtained. Corresponding values given in table V for P-450LM$_2$ of the same publication are 78 and 110, respectively. Corrected extinctions for reduced untreated and MC-treated microsomes (78 and 79, respectively) were essentially identical with those observed for PB-treated microsomes and for P-450LM$_2$. However, the corrected extinctions of reduced + CO/untreated, PB-treated, and MC-treated microsomes (101, 106, and 116, respectively) were less consistent. The extinction coefficients of the Soret maxima of reduced

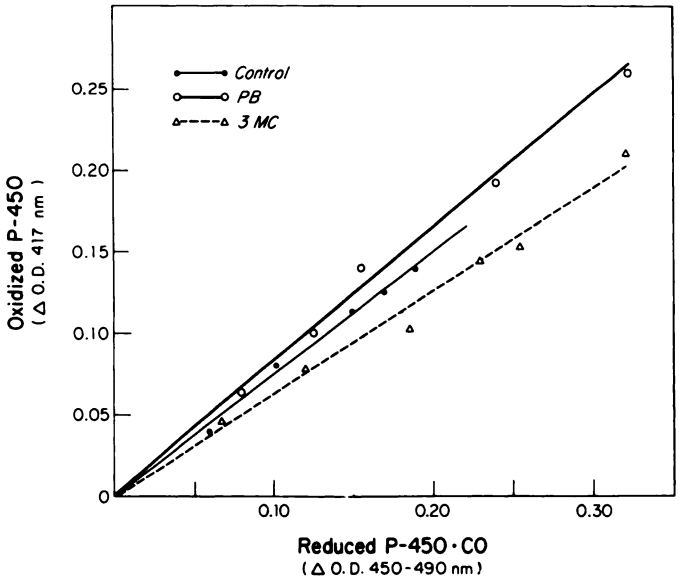

FIG. 2. *Maximal absorbance of oxidized cytochrome P-450 of microsomes from untreated, PB-treated, and MC-treated rats at different levels of LAHP-induced deletion of cytochrome P-450*

The absorbance at 417 of oxidized microsomes in the sample cuvette was determined after each successive addition of LAHP to the oxidized microsomes in the reference cuvette. The concentration of cytochrome P-450 remaining after each addition of LAHP was determined by the method of Omura and Sato (12). The microsomes from untreated, PB-treated, and MC-treated rats contained 0.96, 2.37, and 1.71 nmoles of P-450 hemoprotein per milligram of protein. The concentration of protein in all cuvettes was 2.2 mg/ml (0.1 M phosphate buffer, pH 7.4).

FIG. 1. *Absolute spectra of oxidized, reduced, and reduced carbon monoxide-complexed cytochrome P-450 in microsomes from untreated, PB-treated, and MC-treated rats*

The spectra were obtained using the LAHP deletion procedure described under Methods. Concentrations of cytochrome P-450 in reference and sample cuvettes, respectively (nanomoles of P-450 per milligram of protein), were as follows: A, 0.17 and 0.83; B, 0.57 and 2.3; C, 0.34 and 1.82. The concentration of protein in all cuvettes was 2 mg/ml (0.1 M phosphate buffer, pH 7.4).

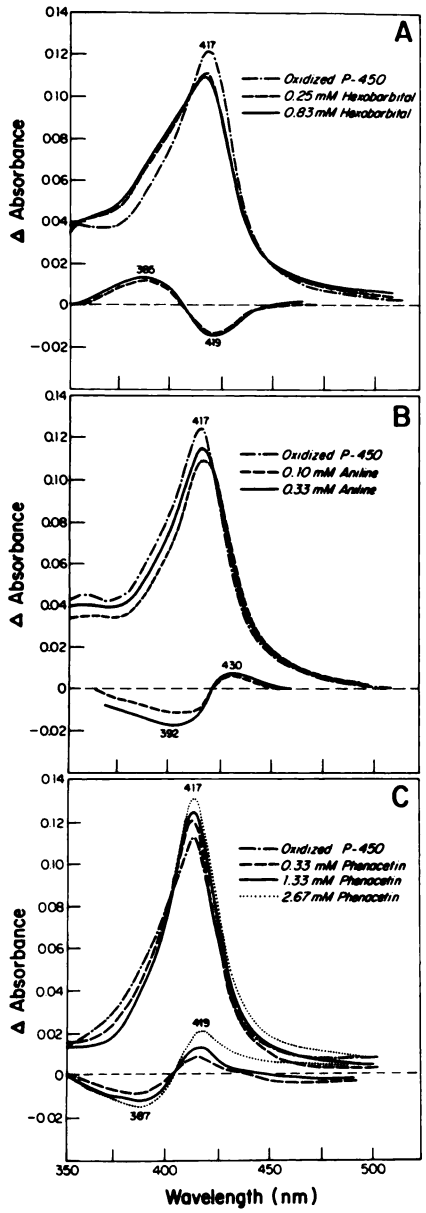

FIG. 3. *Absolute spectra of membrane-bound cytochrome P-450 complexes with Type I (hexobarbital), Type II (aniline), and reverse Type I (phenacetin) compounds*

The observed spectra (upper set of curves in A, B and C) were obtained using oxidized microsomes and the LAHP deletion procedure described under methods; the calculated difference spectra (*lower* set of curves in A, B and C) were obtained by subtracting spectra obtained in the absence of hexobarbital, aniline, or phenacetin from the spectra obtained in the presence of each of these compounds. Concentrations of cytochrome P-450 in reference and sample cuvettes, respectively (nanomoles of P-450 per milligram of protein) were as follows: A, 0.11 and 0.82; B, 0.11 and 0.82; C, 0.26 and 0.88. The concentration of protein in all cuvettes was 2 mg/ml (0.1 M phosphate buffer, pH 7.4). Microsomes were obtained from untreated rats.

and reduced + CO cytochrome P-450$_{cam}$ (76.7 and 121) reported by Gunsalus and Wagner (22) are also reasonably close to those observed in the current study and by Haugen and Coon (15) for P-450LM$_2$, although the λ_{max} of each is 4 or 5 nm lower than those observed for the mammalian cytochrome P-450s. Ryan and associates (18) have recently reported spectra and extinction coefficients for three cytochrome P-450s, designated P-450a, P-450b and P-450c, which they isolated from hepatic microsomes of rats treated with the polychlorinated biphenyl mixture, Aroclor 1254. The extinctions of the reduced-CO Soret maxima of P-450a, P450-b, and P-450c (116, 114, and 117), were in good agreement with those observed in the current study. However, corresponding reduced spectra of the three species of P-450 (100, 85, and 98) were considerably higher than that observed in the current study and for P-450LM$_2$. The reason for this discrepancy is not apparent, but it would seem likely that differences in methodology could account for the disparity (e.g., the P-450a preparation contained 20% glycerol, and the P-450b and P-450c preparations contained added EDTA, cholate, and Emulgen 911).

The extinctions of the Soret maxima of the oxidized microsomes were considerably lower than those reported for solubilized cytochrome P-450 preparations. This can be explained by the presence of the high-spin form of cytochrome P-450 in the membrane. The Soret maximum of low-spin cytochrome P-450 is at 418 nm, that of high-spin cytochrome P-450, at 393 nm. The spectrum of oxidized P-450LM$_2$ is a typical low-spin spectrum. On the other hand, the membrane-bound cytochrome P-450 exists in both high- and low-spin states, and the observed absolute spectrum reflects the mixture of the two forms. Complexes of cytochrome P-450 with unidentified endogenous substrates or membrane components could account for the high-spin state of membrane-bound cytochrome P-450; presumably, solubilization and purification of cytochrome P-450 dissociates these complexes and returns the cytochrome to its low-spin state.

Several species of P-450-hemoprotein exist in hepatic microsomes (15–18). Reproducible absolute spectra can be obtained by the LAHP deletion technique at every level of destruction of cytochrome P-450 only if each of the species of cytochrome P-450 has the same spectral characteristics or if not, if each of the cytochromes that are susceptible to destruction by the hydroperoxide are destroyed equally. That one or the other of these possibilities is the case was shown by the consistency of the extinction of the maximal absorbance of the oxidized absolute spectrum over a wide range of LAHP-induced destruction of cytochrome P-450 (Fig. 2). This was seen regardless of the source of microsomes, whether from untreated rats or from rats treated with phenobarbital or 3-methylcholanthrene.

The same absolute spectra were observed with untreated and PB-treated microsomes when the LAHP deletion technique was used. This affirms the reliability of the Kinoshita-Horie technique (1) when untreated and PB-treated microsomes are balanced against each other spectrally to take advantage of the difference in the inducibilities of cytochrome P-450 and cytochrome b_5 by phenobarbital. On the other hand, the absolute spectra observed with MC-treated microsomes are different from those obtained with untreated and PB-treated microsomes owing to the induction of cytochrome P$_1$-450. The maximal absorbance of the absolute reduced cytochrome P$_1$-450-CO spectrum is 448 nm (Fig. 1, Table 1), which is the same as that observed for the absolute spectrum of

purified preparations of this hemeprotein (23) or for the difference spectrum of MC-treated microsomes obtained in the usual manner (24). The Kinoshita-Horie procedure yields a distorted absolute spectrum with a maximal absorbance at 446 nm (25).

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