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Organic Solvent Extraction of Liver Microsomal Lipid

II. Effect on the Metabolism of Substrates and Binding Spectra of Cytochrome P-450

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SUMMARY

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Extraction of lyophilized liver microsomes from 3-methylcholanthrene- or phenobarbital-treated rats with 1-butanol and acetone removed all the neutral lipids and 80% of the phospholipids; extraction with acetone alone removed all the neutral lipids but only trace amounts of the phospholipids. Recoveries of cytochrome P-450 and NADPH-cytochrome *c* reductase were 80-90%. Using this extraction procedure we examined several properties of the liver microsomal hydroxylating enzyme system and report the following. (a) Extraction of microsomes with 1-butanol and acetone decreased both 3,4-benzpyrene hydroxylase and benzphetamine *N*-demethylase activities. Addition of synthetic phosphatidylcholine to the extracted microsomes restored enzymatic activity to control levels. (b) Extraction of microsomes from phenobarbital-treated rats with 1-butanol and acetone increased the magnitude of both the hexobarbital- and benzphetamine-induced type I binding spectra and the ethanol- and phenacetin-induced reverse type I binding spectra. (c) Extraction of microsomes from 3-methylcholanthrene-treated rats with 1-butanol and acetone eliminated the hexobarbital-induced reverse type I binding spectra. (d) Extraction of microsomes from phenobarbital-treated rats with acetone greatly decreased the initial fast phase in the reduction kinetics of NADPH-dependent cytochrome P-450 reductase. Addition of 1 mM benzphetamine to the extracted microsomes restored the initial fast phase. These results indicate that while lipid is required for enzymatic activity, it is not essential for the binding of substrates to cytochrome P-450. In addition, the phenacetin- and ethanol-induced reverse type I binding spectra are not due simply to the displacement of endogenous substrates from cytochrome P-450.

INTRODUCTION

The mixed-function oxidase system metabolizes not only drugs and other xenobiotics, but also a variety of normal body substrates (1, 2). Diehl *et al.* (3) have calculated that 10-20% of the total cytochrome P-450 present in isolated microsomes is bound to endogenous substrates. These endogenous substrates are thought to be responsible for several phenomena observed in microsomes, i.e., the reverse type I binding spectra (4-7), the fast phase detected in the reduction kinetics of cytochrome P-450 (3), and a part of the endogenous NADPH oxidation (8, 9).

The endogenous substrates may also inhibit the metabolism and binding of added substrates *in vitro*.

The NADPH-dependent mixed-function oxidase system of liver microsomes has been resolved into three components (10–12): cytochrome P-450, NADPH–cytochrome *c* reductase, and a lipid identified as phosphatidylcholine (13). Although the lipid has been shown to be required for the enzymatic reduction of cythchrome P-450 (10), several studies suggest that microsomal lipid may also play a role in the binding of substrates to cytochrome P-450. Removal of phospholipids from microsomes either by treatment with phospholipase (14, 15) or by extraction with isooctane (16) eliminated the type I binding spectra and inhibited the metabolism of type I substrates.

We recently reported (17) that extraction of lyophilized microsomes with 1-butanol and acetone at −20° removes all the neutral lipid, 80% of the phospholipid, and, presumably, lipid-soluble endogenous substrates. Recoveries of cytochrome P-450 and NADPH–cytochrome *c* reductase are approximately 80%. In order to clarify the role of microsomal lipids and endogenous substrates in the mixed-function oxidase system, we have examined the effect of this extraction procedure on the metabolism and binding of several substrates, the RI[1] binding spectra, and the NADPH-dependent reduction of cytochrome P-450.

The results presented in this paper indicate that while lipid is required for enzymatic activity, it is probably not essential for substrate binding. Several lines of evidence demonstrate the removal of endogenous substrates by solvent extraction, and indicate that the RI binding spectra is not due simply to the displacement of endogenous substrates.

MATERIALS AND METHODS

Male, Long-Evans rats (180–200 g) (Blue Spruce Farms, Altamont, N. Y.) were treated intraperitoneally with either phenobarbital (80 mg/kg/day) or 3-methylcholanthrene (25 mg/kg/day) in corn oil for 3 days. Livers were homogenized in 4 volumes of 1.15% KCl, and the 10,000 × *g* supernatant fraction was centrifuged at 105,000 × *g* for 60 min. The microsomal pellet was washed in 1.15% KCl, suspended in distilled H_2O (twice the liver weight), and lyophilized. The evacuated flasks were flushed with argon and stored in a desiccator at −20° for a maximum of 1 week.

Extraction of lipid from microsomes. In a typical experiment, 250 mg of lyophilized microsomes (120 mg of protein) were homogenized in 25 ml of 1-butanol and centrifuged at 35,000 × *g* for 5 min. The pellet was rinsed twice with 25 ml of acetone, and the final acetone suspension was filtered on a Büchner funnel. The fine, cream-colored powder was dried under N_2 and then in a desiccator under vacuum at 4° for 30 min. In some experiments, as noted, microsomes were extracted three to five times with 1-butanol followed by two acetone washings, or extracted a total of three times with acetone alone. The centrifuge and all solvents were kept at −20°, and the entire procedure was carried out at 4°. The procedure must be carried out under strictly anhydrous conditions for minimal loss of cytochrome P-450. The dried powder was homogenized in 20 ml of 0.1 M potassium phosphate buffer, pH 7.7, and sonicated for three 5-sec intervals. Unextracted microsomes were prepared by homogenizing 250 mg of lyophilized microsomes in 20 ml of the buffer and sonicated similarly. Sonication served to keep the microsomes in suspension and was essential for consistent reactivation of enzymatic activity upon the addition of lipid. Appropriate dilutions were then made for individual experiments.

Lipid analysis of microsomes and organic solvent extracts. The lipid contents of the unextracted and extracted microsomes and of the 1-butanol and acetone extracts were analyzed by thin-layer chromatography. Details of the methodology are described in the legends to the table and figures.

Assays. Hydroxylation of 3,4-benzpyrene was assayed by the method of Nebert and Gelboin (18) with slight modification (12). Benzphetamine and ethylmorphine *N*-de-

[1] The abbreviations used are: RI, reverse type I; 3-MC, 3-methylcholanthrene; PB, phenobarbital.

methylase activities were determined by measuring formaldehyde formation by the method of Nash (19) as modified by Cochin and Axelrod (20). Cytochrome P-450 was determined as described by Omura and Sato (21), and protein by the method of Lowry *et al.* (22). Difference spectra were recorded using an Aminco DW-2 dual-wavelength spectrophotometer as previously described (23). Reduction of cytochrome P-450 by NADPH was followed anaerobically in the presence of carbon monoxide by recording the increase in optical density between 450 and 490 nm. The method was similar to that of Gigon *et al.* (24).

Materials. 1-Butanol and acetone (pesticide quality and spectroquality, respectively) were purchased from Matheson, Coleman, and Bell; benzphetamine, from Upjohn and Company; hexobarbital, from Winthrop Laboratories; ethylmorphine, from Mallinckrodt; and NADPH and 3,4-benzpyrene, from Sigma Chemical Company. Synthetic phosphatidylcholine (a mixture containing predominantly the dilauroyl glycerol-3-phosphorylcholine and a small amount of the monolauroyl compound) was a gift from Drs. Mike Radtke and Minor J. Coon.

RESULTS

Removal of lipid from microsomes. Unextracted and extracted microsomes and the organic solvent extracts were examined by thin-layer chromatography to determine the effectiveness of 1-butanol and acetone in removing lipid from lyophilized microsomes. Figure 1 shows the chromatograms of unextracted microsomes (*H*), microsomes extracted five times with 1-butanol (*I*), and the five 1-butanol extracts (*A–E*). The first two 1-butanol extracts removed all the neutral lipid and 90% of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE). As many as five 1-butanol extractions removed only 30–40% of the more polar phospholipids, phosphatidylinositol (PI) and phosphatidylserine.

Figure 2 shows the chromatograms of unextracted microsomes (*A*), microsomes extracted three times with acetone (*D*), and the first two acetone extracts (*B* and *C*). Extraction with acetone removed all the neutral lipid but only trace amounts of the phospholipids. Extraction of microsomes once with 1-butanol and twice with acetone (*E*) has been shown to remove all the neutral lipid, 80% of the phosphatidylcholine and phosphatidylethanolamine, 20% of the phosphatidylinositol, and 70% of the total phospholipid phosphorus (17).

Recovery of cytochrome P-450. Cytochrome P-450 was quite stable to organic solvent extraction if the procedure was carried out under anhydrous conditions. Any moisture present in the microsomes as a result of poor lyophilization or contamination from the air resulted in a loss of cytochrome P-450. Recovery of cytochrome P-450 after one 1-butanol and two acetone extractions ranged from 70 to 85%, while recovery after three 1-butanol and two acetone extractions was 50–60%. Cytochrome P-448 from 3-MC-treated rats was more stable than cytochrome P-450 from PB-treated rats. Recovery of cytochrome P-450 after three acetone extractions was 80–90%. Acetone extraction did not result in the formation of P-420, as was the case with 1-butanol extraction.

Hydroxylation of 3,4-benzpyrene. The enzymatic activity of microsomes from 3-MC-treated rats after one 1-butanol and two acetone extractions was decreased to 40% of the activity of unextracted microsomes as measured by 3,4-benzpyrene hydroxylase activity (Fig. 3). An excess of synthetic phosphatidylcholine restored full activity to the extracted microsomes at all protein concentrations. We have previously shown that both synthetic phosphatidylcholine and total lipid extracts of microsomes will reactivate extracted microsomes, and that the extent of reactivation is dependent on the concentration of lipid used (17).

N-Demethylation of ethylmorphine and benzphetamine. Table 1 shows the effect of one or three 1-butanol and two acetone extractions of microsomes from PB-treated rats on ethylmorphine and benzphetamine *N*-demethylase activities. Differential effects were obtained with the two substrates. The benzphetamine *N*-demethylase activity was increased 20% and decreased 25% by one and three 1-butanol extractions, respectively,

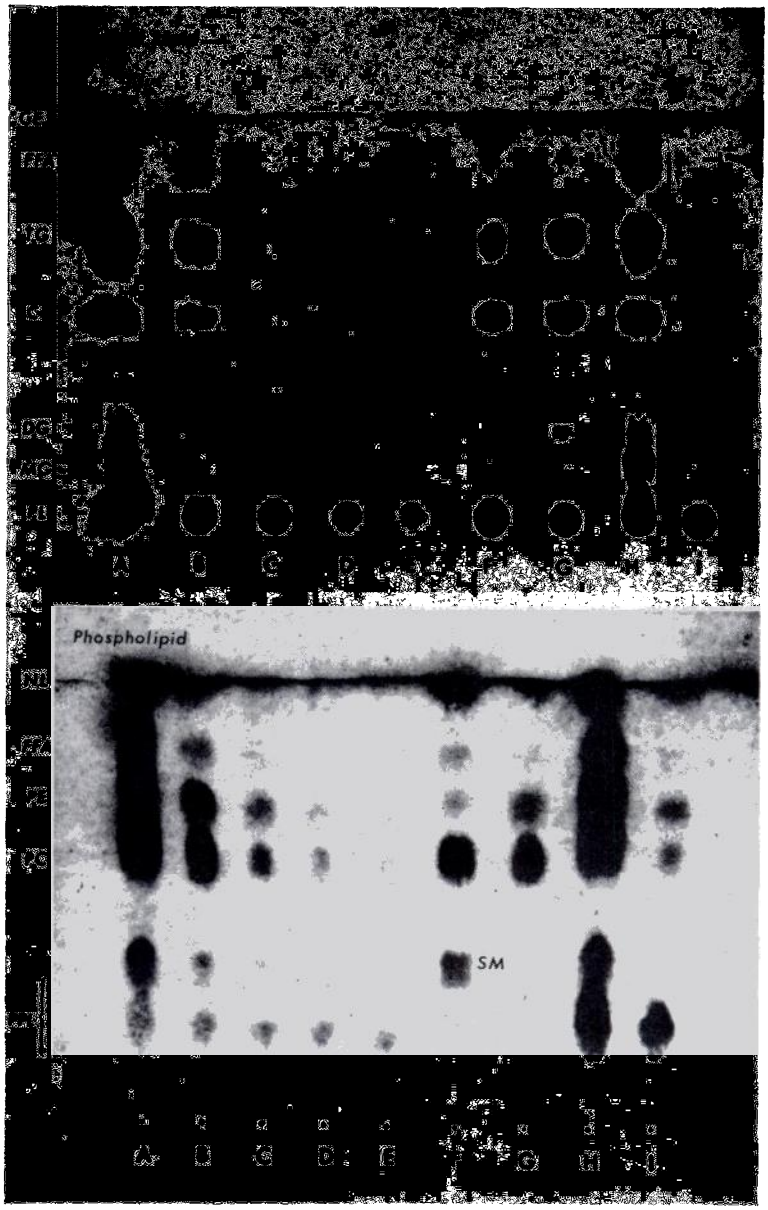

FIG. 1. *Chromatograms of neutral lipids (upper) and phospholipids (lower) present in unextracted microsomes (H), microsomes extracted five times with 1-butanol (I), and each of the five 1-butanol extracts (A–E, respectively)*

Serum lipid (*F*) and egg yolk lipid (*G*) were used as standards. Chloroform–methanol (2:1) extracts of both unextracted and extracted microsomes and a comparable aliquot of the 1-butanol extracts were spotted on silica gel-impregnated glass filter paper (Gelman Instrument Company). Neutral lipids were chromatographed in an isooctane–acetic acid (200:3) solvent system, and phospholipids, in a chloroform–methanol–7 N ammonia (200:25:3) solvent system. Chromatograms were visualized by spraying with H_2SO_4 and charring. CE, cholesterol esters; FFA, free fatty acids; TG, triglycerides; C, cholesterol; DG, diglycerides; MG, monoglycerides; PL, phospholipids; NL, neutral lipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PPL, polar phospholipids.

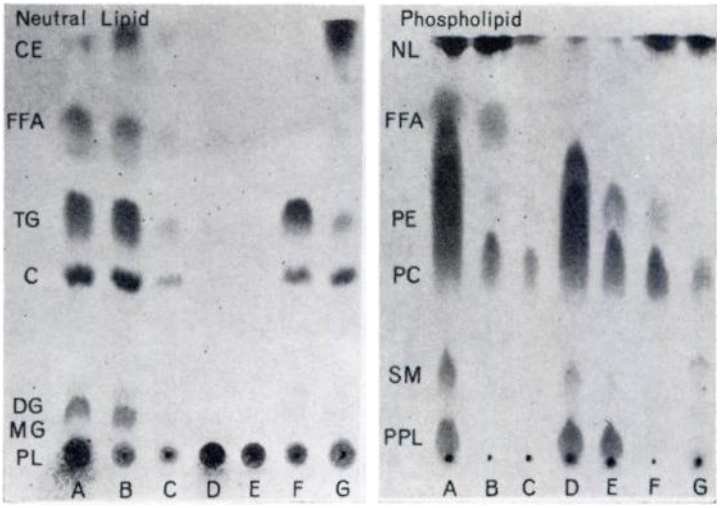

FIG. 2. *Chromatograms of neutral lipids (left) and phospholipids (right) present in unextracted microsomes (A), microsomes extracted three times with acetone (D), the first (B) and second (C) acetone extracts, and microsomes extracted once with 1-butanol and twice with acetone (E)*

Egg yolk lipid (*F*) and serum lipid (*G*) were used as standards. Chromatograms were developed in the same manner as described for Fig. 1. CE, cholesterol esters; FFA, free fatty acids; TG, triglycerides; C, cholesterol; DG, diglycerides; MG, monoglycerides; PL, phospholipids; NL, neutral lipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PPL, polar phospholipids.

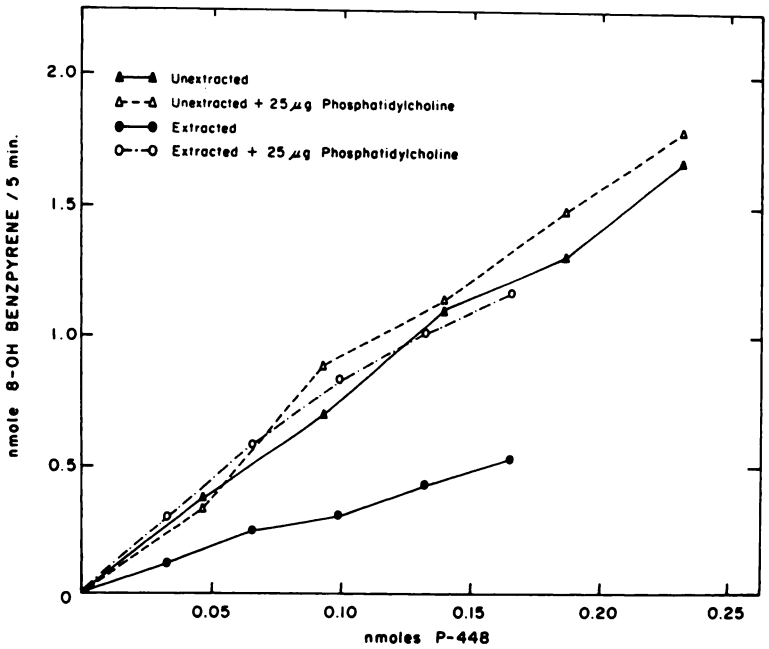

FIG. 3. *Reactivation of 3,4-benzpyrene hydroxylase activity of 1-butanol- and acetone-extracted microsomes from 3-MC-treated rats by addition of synthetic phosphatidylcholine*

The reaction mixture (1 ml) contained 100 μmoles of potassium phosphate buffer (pH 7.3), 3 μmoles of $MgCl_2$, 0.4 μmole of NADPH, 80 nmoles of 3,4-benzpyrene (in 40 μl of acetone), and the indicated amounts of phosphatidylcholine and cytochrome P-448 (1.18 and 0.89 nmoles/mg of protein in unextracted and extracted microsomes, respectively). The reaction mixture was incubated at 37° for 5 min, and 8-hydroxy-3,4-benzpyrene was determined (18, 12).

TABLE 1

Effect of organic solvent extraction on benzphetamine and ethylmorphine N-demethylation

Liver microsomes from PB-treated rats were lyophilized and suspended in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted one or three times with 1-butanol and twice with acetone as described in the text and then suspended in the buffer ("extracted"). The reaction mixture (2 ml) contained 200 μmoles of 0.1 M potassium phosphate buffer, pH 7.3; 7 μmoles of $MgCl_2$; 7 μmoles of semicarbizide; 2 μmoles of EDTA; 2 μmoles of NADP; 10 μmoles of glucose 6-phosphate; 1.4 units of glucose 6-phosphate dehydrogenase; 2 μmoles of benzphetamine or 20 μmoles of ethylmorphine; the indicated amount of synthetic phosphatidylcholine; and 0.3–0.6 mg of protein (0.5 nmole of cytochrome P-450). The reaction mixture was incubated at 37° for 10 min, and formaldehyde was determined (19, 20).

Preparation	Benzphetamine	Ethylmorphine
	nmoles HCHO/min/nmole P-450	
Unextracted microsomes	11.0	12.5
Extracted microsomes		
One 1-butanol extraction	13.1	6.1
+ 50 μg of phosphatidylcholine	13.9	5.9
Three 1-butanol extractions	8.1	3.1
+ 50 μg of phosphatidylcholine	12.0	3.1

while the *N*-demethylation of ethylmorphine was decreased 50% and 75% by one and three 1-butanol extractions, respectively. Addition of synthetic phosphatidylcholine fully restored the benzphetamine *N*-demethylase activity, but had no effect on ethylmorphine *N*-demethylation. The reasons for these differential effects are not presently understood. The data suggest that two different forms of cytochrome P-450 may be required for the *N*-demethylation of benzphetamine and ethylmorphine and that the cytochrome P-450 required for the *N*-demethylation of ethylmorphine either was destroyed by the extraction procedure or requires different conditions for reactivation. Several previous observations made in this laboratory support the suggestion of two different forms of cytochrome P-450 for ethylmorphine and benzphetamine *N*-demethylation. (a) In microsomes from PB-treated, immature male Long-Evans rats, benzphetamine *N*-demethylation is more sensitive to inhibition by β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) than is ethylmorphine *N*-demethylation.[2] (b) Prior treatment of rats with pregnenolone 16α-carbonitrile preferentially induces ethylmorphine *N*-demethylation over benzphetamine *N*-demethylation (25). (c) Ethylmorphine *N*-demethylase activity is approximately 50% higher than benzphetamine *N*-demethylase activity in microsomes from PB-treated, immature male Long-Evans rats. However, after partial purification of the cytochrome P-450, ethylmorphine *N*-demethylase activity is only 30% of benzphetamine *N*-demethylase activity, indicating a preferential loss or inhibition of the former (26).

Hexobarbital and benzphetamine-induced binding spectra in microsomes from PB-treated rats. Previous studies had shown that the type I binding spectra obtained with hexobarbital and benzphetamine were greatly decreased when 70–80% of the lipids were removed (14–16). We therefore examined the effect of extraction of lipid with 1-butanol and acetone on the binding properties of these substrates. In contrast to the earlier reports, typical type I binding spectra were observed in the extracted microsomes. The double-reciprocal plots of the hexobarbital- and benzphetamine-induced type I difference spectra (Fig. 4) show that one 1-butanol and two acetone extractions decreased the K_s and increased the ΔA_{max} for both substrates. Similar results were obtained after removal of 90% of the total lipid by four 1-butanol and two acetone extractions. The decreased K_s and increased ΔA_{max} seen in extracted microsomes strongly suggest that lipid is not essential for substrate binding. The data also suggest that more enzyme is available for binding as a result of the removal of endogenous substrates by the extraction procedure. The $\Delta A_{390-420}$ value in each experiment was calcu-

[2] S. West and A. Y. H. Lu, unpublished observations.

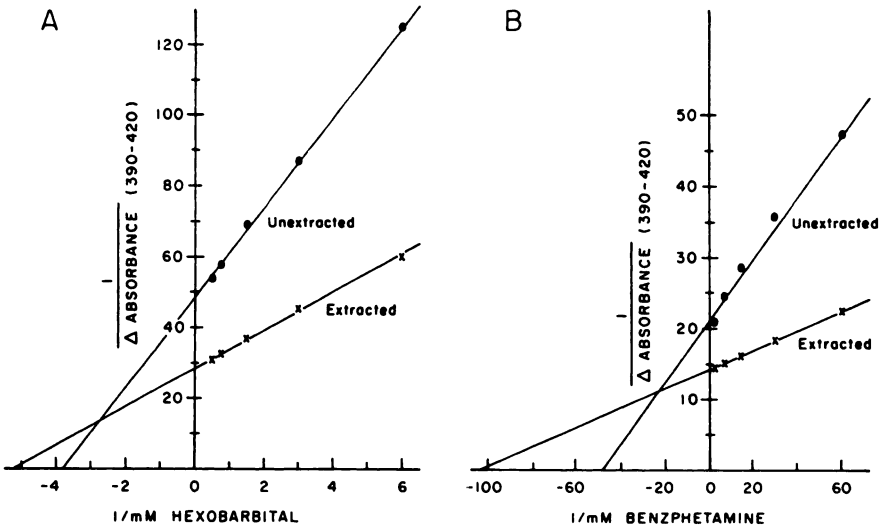

FIG. 4. *Double-reciprocal plots of hexobarbital-(A) and benzphetamine- (B) induced type I spectral change in unextracted and extracted microsomes*

Liver microsomes from PB-treated rats were lyophilized and suspended to 2.0 mg of protein per milliliter in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted once with 1-butanol and twice with acetone as described in MATERIALS AND METHODS and then suspended in the buffer to 2.0 mg of protein per milliliter ("extracted"). Substrate was added to the sample cuvette, and an equal volume of buffer to the reference cuvette. $\Delta A_{390-420}$ was calculated per nanomole of cytochrome P-450 to correct for the loss of cytochrome P-450 due to the extraction procedure.

lated per nanomole of cytochrome P-450 to correct for the loss of cytochrome P-450 due to extraction.

Hexobarbital-induced difference spectra in microsomes from 3-MC-treated rats Hexobarbital causes a type I spectral change at low concentrations (2 mM) but a RI spectral change at high concentrations (10 mM) in liver microsomes from rats treated with polycyclic hydrocarbons (5, 6). This conversion of a type I to a RI binding spectra is not observed in microsomes from untreated or PB-treated rats. The RI binding spectrum is attributed to the displacement by high concentrations of hexobarbital of polycyclic hydrocarbons retained in the microsomes (4). As shown in Fig. 5, one 1-butanol and two acetone extractions prevented the concentration-dependent conversion of a type I to a RI binding spectra. The elimination of RI binding by high concentrations of hexobarbital could be due to the removal of the polycyclic hydrocarbons or other endogenous substrates by organic solvent extraction. The binding spectra were obtained in the presence of 25% glycerol, since in its absence the addition of hexobarbital caused an atypical spectrum (410 nm peak, 430 nm trough) in extracted microsomes. Even at high concentrations of hexobarbital (10 mM), however, this spectrum did not shift to a RI binding spectrum.

Ethanol- and phenacetin-induced RI binding spectra. The RI binding spectra has been shown to be the result of interaction of a class of compounds with the protein moiety of cytochrome P-450 (5). It is generally assumed that compounds which cause the RI spectral change (e.g., methanol, ethanol, 1-butanol, acetone, and phenacetin) act by displacing endogenous substrates from cytochrome P-450.

We examined the effect of organic solvent extraction on the RI binding spectra, since the above evidence (Figs. 4 and 5) indicated that it was effective in removing endogenous substrates. If the ethanol- and phenacetin-induced RI binding spectra are due to the displacement of endogenous substrates, then organic solvent extraction should re-

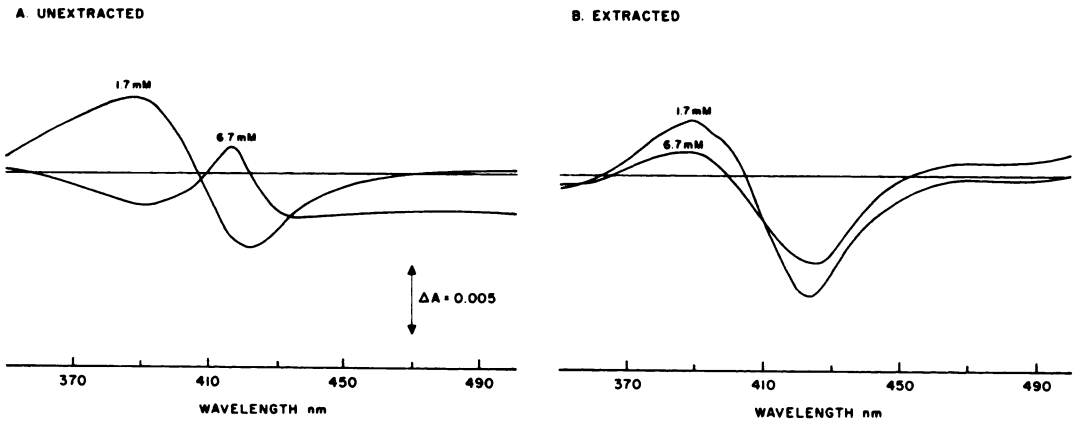

FIG. 5. *Hexobarbital-induced type I and RI spectral changes in unextracted (A) and extracted (B) microsomes*

Liver microsomes from 3-MC-treated rats were lyophilized and suspended to 1.5 mg of protein per milliliter (2.0 nmoles of cytochrome P-448 per milliliter) in 0.1 M potassium phosphate buffer, pH 7.7, containing 25% glycerol ("unextracted"), or were extracted once with 1-butanol and twice with acetone as described in MATERIALS AND METHODS and suspended to 1.5 mg of protein per milliliter (1.7 nmoles of cytochrome P-448 per milliliter) in the buffer ("extracted"). Hexobarbital (0.1 M) was added to the sample cuvette, and an equal volume of buffer to the reference cuvette.

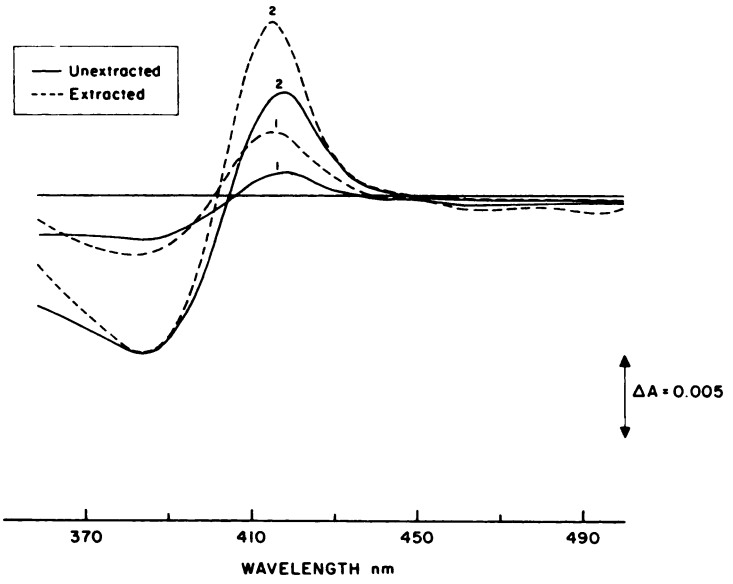

FIG. 6. *Ethanol-induced RI spectral change in unextracted and extracted microsomes*

Liver microsomes from 3-MC-treated rats were lyophilized and suspended to 1.5 nmoles of cytochrome P-448 per milliliter (1.1 mg of protein per milliliter) in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted once with 1-butanol and twice with acetone as described in MATERIALS AND METHODS and suspended to 1.5 nmoles of cytochrome P-448 per milliliter (1.7 mg of protein per milliliter) in the buffer ("extracted"). Ethanol was added to the sample cuvette, and an equal volume of buffer to the reference cuvette. Curves *1*, 60 mM ethanol; curves *2*, 300 mM ethanol.

sult in a decreased RI binding. However, one 1-butanol and two acetone extractions increased the ethanol-induced RI binding spectra in microsomes from both 3-MC-treated rats (Fig. 6) and PB-treated rats (not shown). The phenacetin-induced RI spectra were increased at low phenacetin concentrations but decreased at high concen-

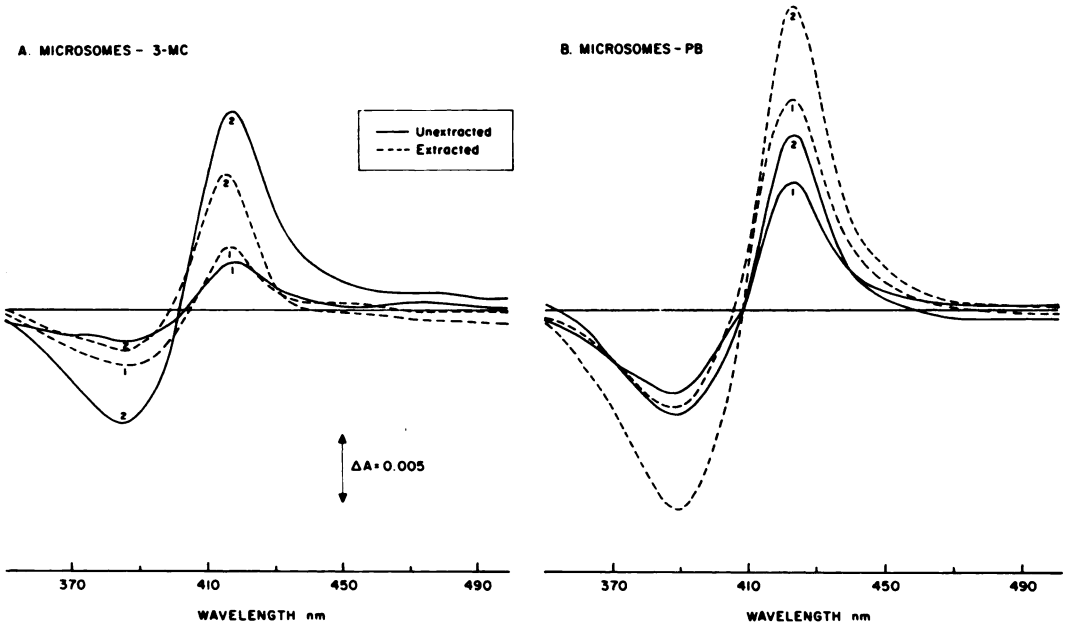

FIG. 7. *Phenacetin-induced RI spectral change in unextracted and extracted microsomes*

Liver microsomes from 3-MC- (A) or PB-(B) treated rats were lyophilized and suspended in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted once with 1-butanol and twice with acetone as described in MATERIALS AND METHODS and then suspended in the buffer ("extracted"). Microsomal suspensions from 3-MC-treated rats contained 1.5 nmoles of cytochrome P-448 per milliliter and 1.1 and 1.7 mg of protein per milliliter in unextracted and extracted preparations, respectively; those from PB-treated rats contained 1.7 nmoles of cytochrome P-450 per milliliter and 1.0 and 1.9 mg of protein per milliter in unextracted and extracted preparations, respectively. Phenacetin in acetone was dried on a stirring rod and stirred into the sample cuvette. Curves *1*, 0.3 mM phenacetin; curves *2*, 1.3 mM phenacetin.

trations in extracted microsomes as compared with unextracted microsomes from 3-MC-treated rats (Fig. 7A). In extracted microsomes from PB-treated rats, however, the RI binding spectra were increased at all phenacetin concentrations (Fig. 7B). Cytochrome P-420, formed by extraction of microsomes in the presence of water, did not give a RI binding spectrum. In each experiment the samples of unextracted and extracted microsomes contained equal micromolar concentrations of cytochrome P-450.

NADPH-dependent reduction of cytochrome P-450. Diehl *et al.* (3) concluded that the initial fast phase in the NADPH-dependent reduction of cytochrome P-450 in microsomes is due to an endogenous cytochrome P-450–substrate complex, and that 10–20% of the total cytochrome P-450 is in the substrate-bound form. As shown in Fig. 8, acetone extraction of microsomes from PB-treated rats markedly decreased the initial fast phase in the reduction of cytochrome P-450. Addition of 1 mM benzphetamine to the extracted microsomes restored the initial fast phase of reduction. Acetone was used in order to remove only the neutral lipid, since phospholipid has been shown to be essential for NADPH-dependent reduction of cytochrome P-450 (13). Equal concentrations of cytochrome P-450 were used in each experiment.

DISCUSSION

Fleischer *et al.* (27) have set forth three criteria for establishing the lipid requirement of enzymes: (a) a decrease in enzymatic activity upon removal of lipid, (b) a reactivation of the enzyme by the addition of lipid, and (c) evidence that the lipid does in fact bind to the enzyme. The data presented here clearly meet the first two requirements

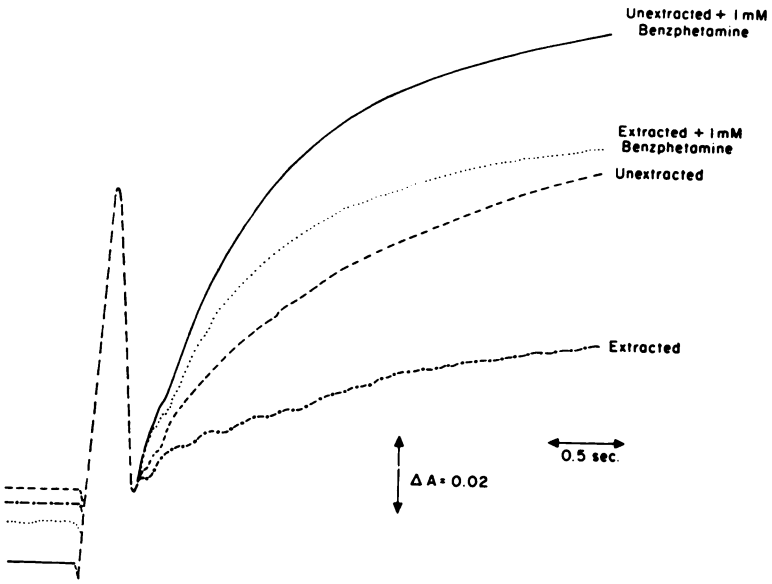

FIG. 8. *Recorder trace of NADPH-dependent anaerobic reduction of cytochrome P-450 in the presence of carbon monoxide in unextracted and extracted microsomes in the presence and absence of substrate*

Liver microsomes from PB-treated rats were suspended in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or extracted three times with acetone as described in MATERIALS AND METHODS and then suspended in the buffer ("extracted"). Microsomal suspensions (2.5 ml) contained 2.18 nmoles of cytochrome P-450 per milliliter and 1.0 amd 1.2 mg of protein per milliliter in unextracted and extracted preparations, respectively. The procedure was carried out at room temperature as described previously (24), except that the plunger contained 10 μl of 0.05 M NADPH.

and show that lipid is required for both the aromatic hydroxylation of 3,4-benzpyrene and the *N*-demethylation of benzphetamine. Evidence that the phosphatidylcholine actually binds to the enzyme requires a preparation of purified cytochrome P-450.

The increased binding of hexobarbital and benzphetamine in extracted microsomes strongly suggests that lipid is not required for substrate binding, although a lipid requirement cannot be completely ruled out since some of the lipid still remains in the microsomes. Additional evidence that lipids are not essential for binding of substrates was obtained in partially purified preparations of cytochrome P-450 which are essentially free of all cholesterol, triglycerides, and phospholipids (28). Addition of various substrates to the lipid-free cytochrome P-450 or P-448 still produces a type I binding spectrum.[3] We cannot explain why treatment of microsomes with phospholipase C (14, 15) or extraction with isooctane (16), which removed no more than 70% of the phospholipid, decreased or eliminated the type I spectra, but it is apparent that it was not simply the removal of lipid which caused the loss of the binding spectra. It was recently reported by Narasimhulu (29) that after a similar extraction of bovine adrenocortical microsomes lipid was required for enzymatic activity but not for substrate binding.

Several lines of evidence are given which suggest that extraction of microsomes with 1-butanol and/or acetone removes endogenous substrates from the microsomes. (a) Acetone extraction of microsomes markedly decreased the initial fast phase in the NADPH-dependent reduction of cytochrome P-450. (b) Thin-layer chromatographic analysis of the extracted microsomes showed that all neutral lipids, including cholesterol and free fatty acids, which are substrates of cytochrome P-450, were

[3] W. Levin and A. Y. H. Lu, unpublished observations.

removed. (c) Addition of dried portions of the acetone extract to the unextracted microsomes produced an apparent type I binding spectrum.[4] Because of turbidity of the extract, only a shoulder in the 380–390 nm range was visible. However, a trough at 420 nm was prominent and concentration-dependent. (d) 1-Butanol extraction increased the ΔA_{max} of the type I binding spectra. The increase in metabolism of benzphetamine after one 1-butanol extraction (Table 1) could also be due to an increase in "available" cytochrome P-450.

The evidence for the removal of endogenous substrates by extraction, and the increased magnitude of the ethanol- and phenacetin-induced RI spectra in extracted microsomes, are inconsistent with the conclusions of Schenkman *et al.* (5, 6) that the RI spectral change is due to a reversal of the structural state of cytochrome P-450 caused by prior binding of substrates to the enzyme *in vivo*. Their conlcusion was based on three lines of evidence: (a) a decrease in magnitude of the RI spectral change after extraction of the microsomes with organic solvents, (b) a decrease in the initial rate of NADPH-dependent reduction of cytochrome P-450 in the presence of compounds causing a RI spectral change, and (c) an increase in the RI spectral change in the presence of compounds causing a type I spectral change. In our experience, extraction of microsomes with organic solvents (i.e., 1-butanol and acetone) in the presence of water causes a significant conversion of cytochrome P-450 to P-420. Since Schenkman and coworkers did not quantitate the recovery of cytochrome P-450 per milligram of protein after extraction, it is possible that the decreased RI spectral change could have been due to a loss of cytochrome P-450 from the microsomes. The decreased rate of NADPH-dependent reduction of cytochrome P-450 in the presence of RI compounds was not significant until well after the initial fast phase (Fig. 7 of ref. 5). The increased RI spectral change seen in the presence of type I compounds does indicate that type I compounds influence the RI binding spectra.

Our data suggest that the RI spectral changes obtained with different compounds may be caused by different mechanisms. The RI binding spectrum obtained with high concentrations of hexobarbital in microsomes from 3-MC-treated rats appears to be due to the displacement of endogenous substrates, since this RI binding spectrum was eliminated by the organic solvent extraction. The data indicate, however, that the ethanol- and phenacetin-induced RI binding spectra are not dependent on the prior binding of endogenous substrates. It is also possible that the hexobarbital-induced RI spectrum may not be due to the displacement of endogenous substrates and that the extraction procedure caused a conformational change in cytochrome P-448 so that hexobarbital could no longer cause a RI binding spectrum.

CO has been shown to inhibit the endogenous rate of NADPH oxidation, suggesting that a significant portion of the endogenous NADPH oxidation is mediated by cytochrome P-450 (8). In order to determine whether the cytochrome P-450-dependent, NADPH-dependent oxidation is due to the presence of endogenous substrates, we examined the effect of acetone extraction on the endogenous NADPH oxidation in microsomes from PB-treated rats. The rates of NADPH oxidation in an N_2–O_2 (8:1) atmosphere for unextracted and extracted microsomes were 5.1 and 3.4 nmoles/min/nmole of P-450, respectively, while the corresponding rates in CO–O_2 (8:1) were 2.6 and 2.4. Thus, while approximately 50% of the endogenous NADPH oxidation was CO-sensitive, acetone extraction decreased the rate by only 30%, and the rate in extracted microsomes was further inhibited by CO. This suggests two possible explanations: either extraction only partially removed the endogenous substrates from the microsomes; or extraction removed essentially all of the endogenous substrates, but part of the cytochrome P-450-dependent NADPH oxidation was due to autoxidation and was not substrate-dependent. Although these data are preliminary and do not give a definitive answer, they support previous suggestions (8, 9) that endogenous substrates are partially responsible for the cytochrome P-450-mediated endogenous NADPH oxidation.

[4] Unpublished observations.

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