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ORGANIC SOLVENT EXTRACTION OF LIVER MICROSOMAL LIPID

EFFECT ON THE KINETIC PARAMETERS OF BENZO[*a*]PYRENE HYDROXYLASE AND BENZPHETAMINE *N*-DEMETHYLASE ACTIVITY*

MARY VORE and EVA SOLIVEN

Departments of Pharmacology, University of California School of Medicine, San Francisco, CA 94143, U.S.A.

and

University of Kentucky College of Medicine, Lexington, KY 40536, U.S.A.

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Abstract—Extraction of lyophilized hepatic microsomes from untreated rats, once with 1-butanol and twice with acetone, increased benzo[*a*]pyrene hydroxylase and benzphetamine *N*-demethylase activities by 25 per cent; these activities were increased further by the addition of dilauroylglyceryl-3-phosphorycholine (di-12-GPC). More extensive extraction with 1-butanol decreased the activities by 50 per cent; addition of di-12-GPC restored activity to control levels. Kinetic analysis indicated that a single extraction with 1-butanol decreased the apparent K_m for benzo[*a*]pyrene 6-fold, with no change in V_{max}; addition of di-12-GPC had no effect on the apparent K_m or V_{max}. In contrast, a single extraction with 1-butanol of microsomes from 3-methycholanthrene (3-MC)-treated rats had no effect on the apparent K_m or V_{max} for benzo[*a*]pyrene. Lineweaver–Burk plots of benzphetamine *N*-demethylase activity in extracted microsomes from untreated rats and in both unextracted and extracted microsomes from 3-MC-treated rats were non-linear with a marked increase in activity at higher benzphetamine concentrations.

The liver microsomal mixed-function oxidase system catalyzes the hydroxylation of a wide variety of substrates, including drugs, carcinogens, pesticides and endogenous substrates such as steroids and fatty acids [1,2]. This enzyme system has been resolved into three components [3–5] which are essential for activity: cytochrome P-450, NADPH-dependent cytochrome *c* (P-450) reductase, and phosphatidylcholine [6].

We reported previously [7] that extraction of lyophilized microsomes with 1-butanol and acetone removed all neutral lipids and 80 per cent of the phospholipids with only a 20 per cent loss of cytochrome P-450 and NADPH-cytochrome *c* reductase. Benzo[*a*]pyrene hydroxylase and benzphetamine *N*-demethylase activities were decreased in extracted microsomes but could be restored to control levels by the addition of dilauroylglyceryl-3-phosphorylcholine (di-12-GPC), thus confirming the lipid requirement of the mixed-function oxidase system. Several lines of evidence indicated that extraction also removed endogenous ligands of cytochrome P-450 from the microsomes. First, extraction of microsomes with acetone alone (which removed all the neutral lipids and trace amounts of phospholipids) decreased the initial fast-phase in the NADPH-dependent reduction of cytochrome P-450. Second, addition of an aqueous suspension of the acetone extracts to unextracted microsomes caused Type I binding spectra. Third, thin-layer chromatographic analysis of extracted microsomes showed that cholesterol and free-fatty acids, substrates of cytochrome P-450, were removed. Fourth, extraction of microsomes with 1-butanol and acetone increased the peak-to-trough difference of the hexobarbital- and benzphetamine-induced Type I binding spectra, the ethanol- and phenacetin-induced modified Type II binding spectra, and the aniline-induced Type II binding spectra. Finally, extraction of microsomes with 1-butanol and acetone increased the *N*-demethylation of benzphetamine.

Several phenomena observed with microsomes have been attributed to the presence of lipid-soluble endogenous substrates in microsomal membranes, i.e. the initial fast phase detected in the reduction kinetics of cytochrome P-450 [8] and a part of the endogenous NADPH oxidation [9,10]. These endogenous substrates may also inhibit the metabolism of exogenous substrates since addition of a variety of steroids to microsomal incubations *in vitro* has been shown to inhibit the metabolism of hexobarbital, ethylmorphine and *p*-nitroanisole [11,12]. Estradiol-17β and testosterone have also been shown to inhibit competitively the hydroxylation of 7,12-dimethylbenzanthracene in mouse liver microsomes [13].

Therefore, it was of interest to determine if solvent extraction of lyophilized microsomes altered the kinetic parameters of the metabolism of exogenous

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drugs and carcinogens by the mixed function oxidase system. The present studies were designed to clarify the role of membrane lipids and endogenous ligands of cytochrome P-450 in the metabolism of benzo[*a*]pyrene and benzphetamine.

MATERIALS AND METHODS

Male Sprague–Dawley rats (240–260 g) (Simonsen, Gilroy, CA) were killed and the livers were perfused with 1.15 per cent KCl–0.1 M potassium phosphate buffer (pH 7.7) and then homogenized in 2 vol. of the buffer. The microsomes were prepared and lyophilized as described previously [7] and stored under argon at −70° for a maximum of 10 days. In some experiments, rats were treated with 3-methylcholanthrene (3-MC) (20 mg/kg/day, i.p.) in corn oil for 3 days prior to being killed.

Extraction of lipid from microsomes. In a typical experiment, 250 mg of lyophilized microsomes were homogenized in 15 ml of 1-butanol and centrifuged, and the pellet was rinsed twice with 15 ml of acetone. The final acetone suspension was filtered, and the powder was dried with N_2 and then placed in a desiccator at −20° for 30 min. In some experiments, as indicated, microsomes were extracted twice with 1-butanol, followed by two acetone rinses. The solvents were kept at −70° in an acetone and dry-ice bath, and the centrifuge was kept at −15° to −20°. The extracted microsomes were then homogenized in 15 ml of 0.1 M potassium phosphate buffer (pH 7.7) and sonicated for three 5-sec intervals. Unextracted microsomes were homogenized and sonicated similarly.

Studies using [^{14}C]-1-butanol (51 μCi/mole) indicated that more than 99.9 per cent of the 1-butanol was removed from extracted microsomes, and that about 0.1 mmole of 1-butanol remained in the extracted microsomes. The approximate molar ratio of 1-butanol to cytochrome P-450 was 800 in extracted microsomes. (The molar ratios of 1-butanol to cytochrome P-450, used in studies designed to induce spectral changes, are in the order of 10,000–100,000 [8, 14, 15].)

Recovery of cytochrome P-450 was 75–85 per cent following extraction once with 1-butanol and 65–70 per cent following two extractions with 1-butanol. All activities were calculated per nmole cytochrome P-450 to correct for the loss due to the extraction procedure.

In order to determine the effectiveness of 1-butanol at −70° in extracting phosphatidylcholine from microsomes, rats were injected intraperitoneally with 50 μCi [^{3}H]choline (*methyl*-^{3}H) (4.2 Ci/mmole) (New England Nuclear, Boston, MA). Aliquots of unextracted and extracted microsomes were then assayed for a total radioactivity. Microsomes which were extracted once or twice with 1-butanol and twice with acetone, contained 18 and 11 per cent, respectively, of the [^{3}H]phosphatidylcholine in unextracted microsomes, as reported previously for microsomes extracted at −20° [16].

Assay. Hydroxylation of benzo[*a*]pyrene was assayed by the method of Nebert and Gelboin [17], with slight modification [18]. Benzphetamine *N*-demethylase activity was determined by measuring formaldehyde formation by the methods of Nash [19], as modified by Cochin and Axelrod [20]. All reactions were carried out under conditions of linearity with respect to time and protein. Cytochrome P-450 was measured as described by Omura and Sato [21], and protein was measured by the method of Lowry *et al.* [22].

Materials. Acetone and 1-butanol were purchased from Burdick & Jackson (Muskegon, MI). di-12-GPC from Serdary Research Laboratories Inc. (Ontario, Canada) and benzo[*a*]pyrene, 3-methylcholanthrene (3-MC), NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase from the Sigma Chemical Co., St. Louis, MO. Benzphetamine hydrochloride was a gift from Upjohn & Co., Kalamazoo, MI.

Kinetic analysis. Michaelis constants for benzo[*a*]pyrene hydroxylase activity and benzphetamine *N*-demethylase activity were calculated by the method of Wilkinson [23] with the use of a Fortran program of Cleland [24].

RESULTS

Effect of extraction on benzo[a]*pyrene hydroxylase and benzphetamine* N*-demethylase activities.* Extraction of microsomes from untreated rats once with 1-butanol and twice with acetone increased benzo[*a*]pyrene hydroxylase activity (Fig. 1A) and benzphetamine *N*-demethylase activity (Fig. 1B) to 120–130 per cent of their activities in unextracted microsomes. Addition of di-12-GPC to extracted microsomes further stimulated activity to 150 per cent of the activity in unextracted microsomes. Addition of di-12-GPC to unextracted microsomes stimulated activity less than 10 per cent at low concentrations and inhibited activity at higher concentrations. Extraction of microsomes twice with 1-butanol and twice with acetone decreased activity by approximately 50 per cent and addition of lipid restored activity to control levels. Thus, extraction of all neutral lipids and of 80 per cent of the phospholipids increased the activity of cytochrome P-450, whereas more extensive extraction, which removed 90 per cent of the phospholipid, decreased activity. This activity could be restored to control levels by the addition of phosphatidylcholine.

Kinetics of benzo[a]*pyrene hydroxylase activity.* The increased benzo[*a*]pyrene hydroxylase activity in microsomes which had been extracted once with 1-butanol and twice with acetone was investigated further by determining the kinetic parameters of the reaction. Nebert and Gelboin [17] and Hansen and Fouts [25] have shown that the apparent K_m for benzo[*a*]pyrene in microsomes decreases with decreasing enzyme concentrations. The Lineweaver–Burk plots of benzo[*a*]pyrene hydroxylase activity in unextracted and extracted microsomes from untreated (control) rats are shown in Fig. 2, panels A and B. Figure 2A shows the results obtained in experiments using 1 mg/ml of microsomal protein, while Fig. 2B shows those using 0.2 mg/ml of microsomal protein. The apparent K_m obtained in experiments using 1 mg/ml of microsomal protein (75 μM) is in agreement with the value of 74 μM reported by

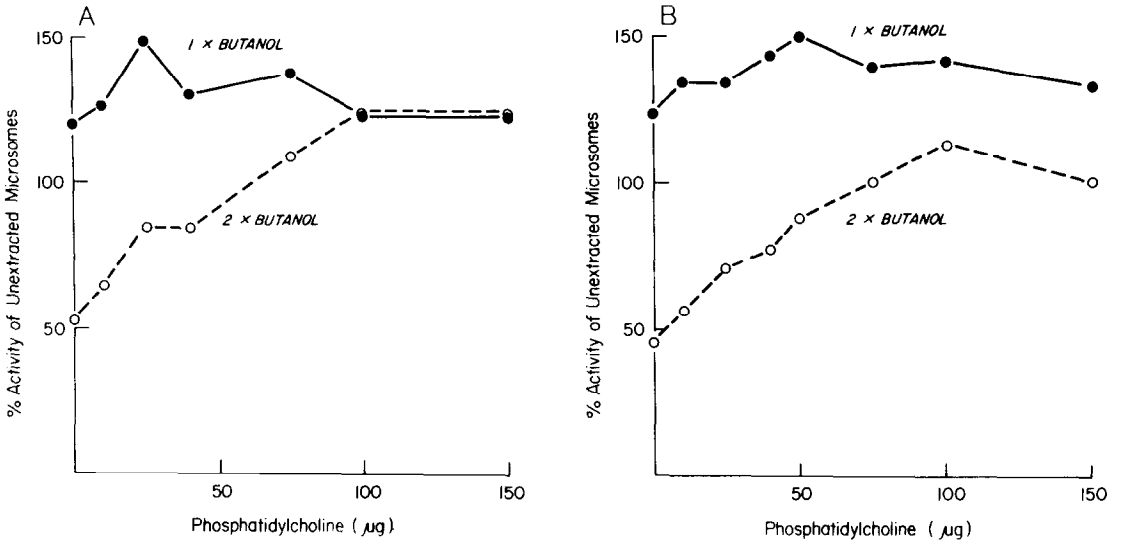

Fig. 1. Effect of extraction of control rat liver microsomes once or twice with 1-butanol and twice with acetone and of di-12-GPC on benzo[*a*]pyrene hydroxylase (A) and benzphetamine *N*-demethylase (B) activities. Panel A: the reaction mixture (1 ml) contained 100 μmoles potassium phosphate buffer (pH 6.8); 3 μmoles $MgCl_2$; 1 μmole EDTA; 1 μmole NADP; 5 μmoles glucose-6-phosphate; 0.7 units glucose-6-phosphate dehydrogenase; 240 nmoles of 3,4-benzo[*a*]pyrene (in 20 μl acetone); the indicated amounts of di-12-GPC and 0.56 or 0.50 nmole cytochrome P-450 in microsomes extracted once or twice with 1-butanol respectively. Unextracted microsomal incubations contained 0.85 nmole cytochrome P-450 and 1.4 mg protein, and extracted microsomal incubations each contained 1.1 mg protein. The reaction mixture was incubated at 37° for 10 min, and 3-hydroxybenzo[*a*]pyrene determined [17, 18]. Activity in unextracted microsomes (100 per cent) was 0.25 nmole of product formed/nmole of cytochrome P-450/min. Panel B: the reaction mixture (2 ml) contained 200 μmoles potassium phosphate buffer (pH 7.4); 7 μmoles $MgCl_2$; 7 μmoles semicarbazide; 2 μmoles EDTA; 2 μmoles NADP; 10 μmoles glucose-6-phosphate; 1.4 units glucose-6-phosphate dehydrogenase; 2 μmoles benzphetamine; the indicated amounts of di-12-GPC and 0.70 or 0.63 nmole of cytochrome P-450 in microsomes extracted once or twice with 1-butanol respectively. Unextracted microsomal incubations contained 1.1 nmoles cytochrome P-450 and 1.8 mg protein, and extracted microsomal incubations each contained 1.4 mg protein. The reaction mixture was incubated at 37° for 10 min and formaldehyde measured [19, 20]. Activity in unextracted microsomes (100 per cent) was 3.3 nmoles of product formed/nmole of cytochrome P-450/min.

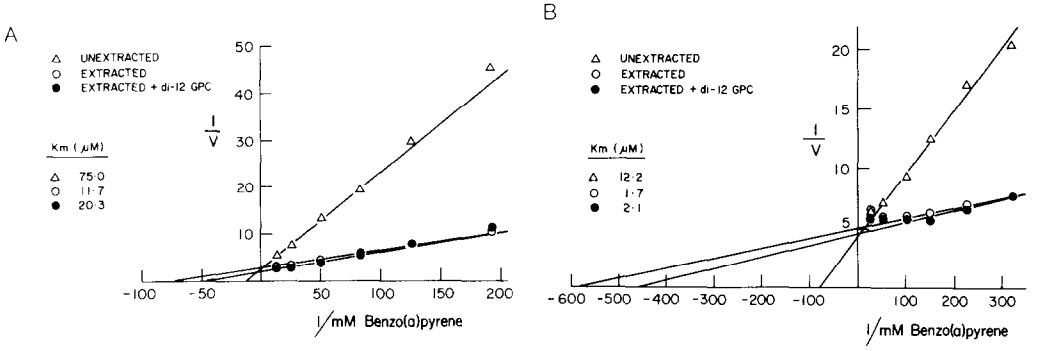

Fig. 2. Lineweaver–Burk plots for benzo[*a*]pyrene hydroxylase activity in unextracted and extracted liver microsomes from control rats using high protein concentrations (1 mg/ml) (A) or low protein concentrations (0.2 mg/ml) (B). The reaction mixture and conditions were as described in the legend to Fig. 1A and contained the indicated amounts of benzo[*a*]pyrene added in 20 μl acetone. The points represent the mean values of duplicate determinations from three (A) or five (B) separate experiments. Velocities were calculated as nmoles of 3-hydroxybenzo[*a*]pyrene formed/nmole of cytochrome P-450/min. Panel A: unextracted microsomes and microsomes extracted once with 1-butanol and twice with acetone contained 0.87 and 0.54 nmole cytochrome P-450, respectively; di-12-GPC (50 μg; 80 nmoles) was added to extracted microsomes. V_{max} values were 0.37, 0.34 and 0.52 in unextracted microsomes, extracted microsomes, and extracted microsomes plus di-12-GPC respectively. Panel B: unextracted microsomes and microsomes extracted once with 1-butanol and twice with acetone contained 0.10 and 0.06 nmole cytochrome P-450, respectively; di-12-GPC (5 μg; 8 nmoles) was added to extracted microsomes. V_{max} values were 0.23, 0.20 and 0.23 in unextracted microsomes, extracted microsomes, and extracted microsomes plus di-12-GPC respectively.

Hansen and Fouts [26], while the apparent K_m obtained in experiments using 0.2 mg/ml of microsomal protein (12.2 μM) is in agreement with the value of 11.4 μM reported by Alvares *et al.* [27]. Regardless of the protein concentration used, extraction of microsomes caused a 6- to 7-fold decrease in the apparent K_m but no change in the V_{max} (Fig. 2, panels A and B). In experiments using 1 mg/ml of microsomal protein, addition of di-12-GPC (50 μg; 80 nmoles) to extracted microsomes had no effect on the apparent K_m but increased the V_{max} by 50 per cent. In experiments using 0.2 mg/ml of microsomal protein, addition of di-12-GPC (5 μg; 8 nmoles) to extracted microsomes had no effect on the apparent k_m and increased the V_{max} by 20 per cent.

The effects of solvent extraction on the kinetic parameters of benzo[*a*]pyrene hydroxylase activity in liver microsomes from 3-MC-treated rats were also investigated. As shown in Fig. 3, in experiments using 0.1 mg/ml of microsomal protein, extraction caused only a 35 per cent decrease in the apparent K_m and no change in the V_{max}. Addition of di-12-GPC (25 μg; 40 nmoles) had no effect on the apparent K_m and increased the V_{max} by 30 per cent. These data are in agreement with our previous studies [7,16] demonstrating that extraction of microsomes from 3-MC-treated rats decreased benzo[*a*]pyrene hydroxylase activity and that this activity was restored to control levels by the addition of di-12-GPC. In experiments using 1 mg/ml of microsomal protein (data not shown), the apparent K_m in unextracted microsomes (19.9 μM) was also decreased by only 25 per cent to 15.1 μM in extracted microsomes.

Thus, extraction of control microsomes decreased the apparent K_m 6- to 7-fold to values comparable to those obtained in 3-MC microsomes, whereas extraction of 3-MC microsomes had essentially no effect on the apparent K_m. Addition of lipid to extracted microsomes from both untreated and 3-MC-treated animals increased the V_{max} by 20–50 per cent but had no effect on the apparent K_m.

Kinetics of benzphetamine N-*demethylase activity.* The Lineweaver–Burk plots of benzphetamine *N*-demethylase activity in unextracted and extracted microsomes from untreated rats are shown in Fig. 4A. Non-linear kinetics were obtained in extracted microsomal preparations with a marked increase in activity at benzphetamine concentrations greater than 0.25 mM. Activity in extracted microsomes was less than that in unextracted microsomes at concentrations of benzphetamine less than 0.25 mM. Addition of di-12-GPC (50 μg; 80 nmoles) to extracted microsomes further increased activity at higher benzphetamine concentrations and restored activity at lower benzphetamine concentrations to that in unextracted microsomes.

Because of the striking similarity between the kinetic parameters of benzo[*a*]pyrene hydroxylase activity in extracted microsomes from untreated rats and microsomes from 3-MC-treated rats, the kinetics of benzphetamine *N*-demethylase activity in 3-MC microsomes were also investigated. As shown in Fig. 4B, non-linear kinetics were also obtained in Lineweaver–Burk plots of benzphetamine *N*-demethylase activity in both unextracted and extracted microsomes. Extraction of microsomes increased activity at all benzphetamine concentrations, and addition of di-12-GPC to extracted microsomes further increased activity.

DISCUSSION

The present studies show clearly that extraction of lyophilized hepatic microsomes from untreated rats once with 1-butanol and twice with acetone

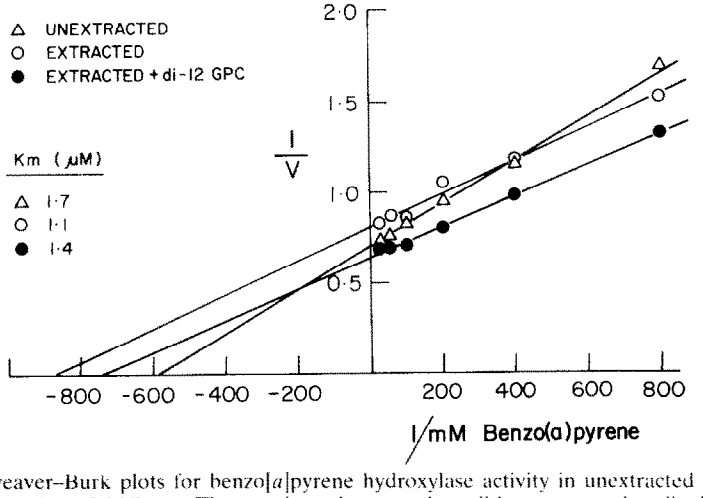

Fig. 3. Lineweaver–Burk plots for benzo[*a*]pyrene hydroxylase activity in unextracted and extracted liver microsomes from 3-MC rats. The reaction mixture and conditions were as described in the legend to Fig. 1A except that the reaction was incubated for 5 min. The reaction mixture contained 0.11 and 0.07 nmole cytochrome P-450 in unextracted and extracted microsomes, respectively, and the indicated amounts of benzo[*a*]pyrene added in 20 μl acetone; di-12-GPC (25 μg; 40 nmoles) was added to extracted microsomes. The points represent the mean values of duplicate determinations in three separate experiments. Velocities were calculated as nmoles of 3-hydroxybenzo[*a*]pyrene formed/nmole of cytochrome P-450/min. V_{max} values were 1.42, 1.23 and 1.60 in unextracted microsomes, extracted microsomes, and extracted microsomes plus di-12-GPC respectively.

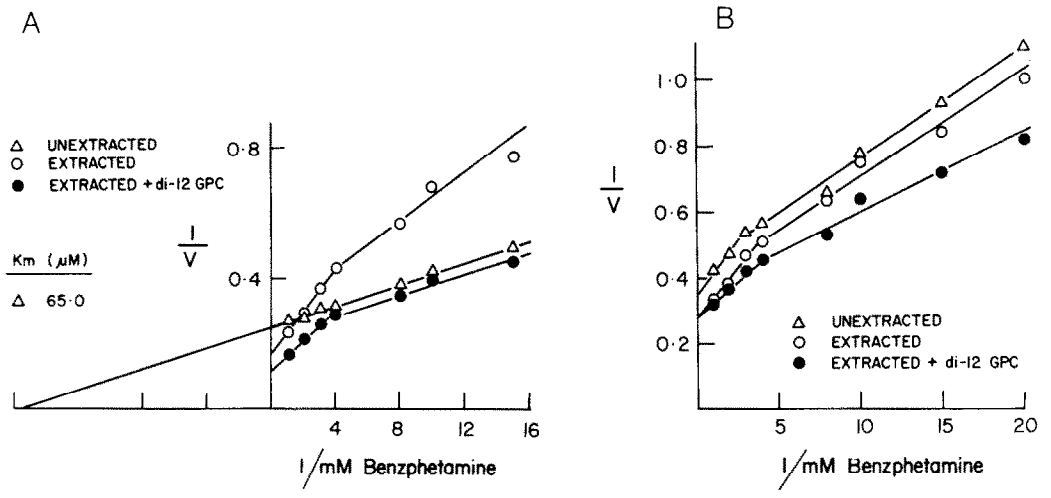

Fig. 4. Lineweaver–Burk plots of benzphetamine *N*-demethylase activity in unextracted and extracted liver microsomes from control rats (A) and from 3-MC-treated rats (B). The reaction mixture and conditions were as described in the legend to Fig. 1B and contain the indicated amounts of benzphetamine; di-12-GPC (50 μg; 80 nmoles) was added to extracted microsomes. The points represent the mean values of duplicate determination from four separate experiments. Velocities were calculated as nmoles of HCHO formed/nmole of cytochrome P-450/min. Panel A: unextracted and extracted microsomes contained 1.1 and 0.64 nmoles cytochrome P-450 respectively; V_{max} in unextracted microsomes was 3.95. Panel B: unextracted and extracted microsomes contained 1.7 and 1.2 nmoles cytochrome P-450 respectively.

increased both benzo[*a*]pyrene hydroxylase and benzphetamine *N*-demethylase activity. More extensive extraction decreased enzymatic activity approximately 50 per cent and this activity could be restored to the levels in unextracted microsomes by the addition of synthetic phosphatidylcholine. These data are in agreement with our previous studies demonstrating the lipid requirement of the mixed-function oxidase system in microsomes from 3-MC- and PB-induced rats.

Extraction of control microsomes decreased the apparent K_m for benzo[*a*]pyrene 6- to 7-fold with no change in the V_{max}, suggesting that extraction had removed a competitive inhibitor of benzo[*a*]pyrene hydroxylase from the microsomal membrane. The kinetic parameters of benzo[*a*]pyrene hydroxylase in microsomes have been investigated extensively, and several laboratories have shown that the apparent K_m in microsomes decreases with decreasing protein concentration [17, 25, 28]. This has been interpreted to indicate that the microsomal membrane contains non-specific binding sites for benzo[*a*]pyrene which compete with the enzyme for the substrate, this mimicking the kinetics of a competitive inhibitor and resulting in falsely high K_m values at higher protein concentrations. Lu *et al.* [29] have shown that the apparent K_m values of liver microsomal epoxide hydrase for several substrates are also dependent on the concentration of microsomal protein used. When purified epoxide hydrase was examined, however, the apparent K_m for benzo[*a*]pyrene-11, 12-oxide was found to be independent of protein concentration but dependent on the concentration of lipid, such that addition of di-12-GPC at concentrations greater than its critical micelle concentration inhibited activity in a competitive manner. These data were found to be consistent with a model in which the substrate is partitioning between non-specific sites in the lipid micelles and the aqueous medium. It is reasonable to suggest that a similar phenomenon is occurring with benzo[*a*]pyrene hydroxylase in control microsomes and that extraction decreases the apparent K_m by removing the lipid.

The lack of effect of extraction on the apparent K_m for benzo[*a*]pyrene in 3-MC microsomes could, therefore, be interpreted to indicate a decrease in the non-specific binding of benzo[*a*]pyrene in the lipid in these microsomes. Decreased non-specific binding could be due to changes in the properties of the membrane lipids after 3-MC treatment, such that alterations in the fluidity of the phospholipid bilayer could either facilitate diffusion of benzo[*a*]pyrene to the substrate binding site of cytochrome P-450 or increase the rate of transfer of benzo[*a*]pyrene from the lipid phase to the substrate binding site. Davison and Wills [30] have shown a 30 per cent decrease in the molar ratio of cholesterol/total phospholipid in liver microsomal membranes 5 days after a single dose of 3-MC (20 mg/kg). Changes in the cholesterol content of membranes have been shown to alter membrane fluidity [31] and could, therefore, contribute to alterations in the apparent K_m. However, these workers [30] demonstrated a similar decrease in the cholesterol content after pretreatment with phenobarbital (PB). PB treatment has been shown to have no effect on the apparent K_m for benzo[*a*]pyrene in rat liver microsomes [32].

It should be pointed out, however, that the apparent K_m in 3-MC microsomes is also increased with increasing protein concentrations to approximately the same extent as that in control microsomes, suggesting that non-specific binding does occur in 3-

MC microsomes. In addition, the apparent K_m in both 3-MC and control extracted microsomes is also dependent on the protein concentration, suggesting that non-specific binding still occurs after removal of the lipid. It is likely that the highly nonpolar benzo[*a*]pyrene binds non-specifically to the hydrophobic membrane proteins as well. Thus, a decrease in the non-specific binding in 3-MC microsomes does not appear to be responsible for the lack of effect of extraction on the apparent K_m for benzo[a]pyrene.

Alternatively, the three-dimensional orientation of cytochrome P-448 in the microsomal membrane after 3-MC induction may be such that the specific substrate binding site is more accessible to benzo[*a*]pyrene. This could result from the increased synthesis of cytochrome P-448 and, therefore, an increased number of specific high affinity benzo[*a*]pyrene binding sites dispersed throughout the membrane. Recently, Thomas *et al.* [33] have used monospecific antibodies to cytochrome P-448 to quantitate this form of cytochrome P-450 in microsomes. Their data demonstrate that, in microsomes from 3-MC-treated rats, 89 per cent of the cytochrome P-450 was immunologically identical to cytochrome P-448, whereas in microsomes from untreated rats, this value was only 6 per cent of the total cytochrome P-450.

A great deal of indirect evidence has accumulated indicating that microsomes from untreated rats [34-37], rabbits [38] and mice [39, 40] contain multiple forms of cytochrome P-450 and that at least one of these forms is inducible by 3-MC. As discussed above, Thomas *et al.* [33] have used a direct method to demonstrate the presence of cytochrome P-448 in control microsomes. Finally, then, the activity of cytochrome P-448 which is present in small amounts in control microsomes and which has a high affinity for benzo[*a*]pyrene may be masked by the presence of competitive inhibitors which are extracted by 1-butanol. The nature of the proposed inhibitors is not known, or if they are, in fact, endogenous substrates. It is unlikely that the inhibitors are free fatty acids or cholesterol, however, since extraction of microsomes with acetone alone, which removes neutral lipids (including free fatty acids and cholesterol) and only trace amounts of phospholipids [7], had no effect on the apparent K_m or V_{max} for benzo[*a*]-pyrene hydroxylase activity. This could indicatethat the inhibitors are either polar in nature or are tightly bound in the phospholipids of the microsomal membrane.

The non-linear kinetics for benzphetamine *N*-demethylase activity obtained in extracted microsomes are difficult to interpret, but suggest that at least two forms of cytochrome P-450 with distinct affinities and lipid requirements are involved in catalyzing this reaction [41]. Similar non-linear kinetics were also obtained in both unextracted and extracted 3-MC microsomes. These data are consistent with the hypothesis that extraction has removed an inhibitor from a form of cytochrome P-450 present in control microsomes with an affinity for benzphetamine similar to that induced by treatment with 3-MC.

Recently, several studies have provided evidence suggesting that 1-butanol and other agents containing a sterically accessible oxygen atom, which induce a reverse Type I (modified Type II) binding spectra, cause the formation of low-spin cytochrome P-450 by binding of the oxygen atom to ferric iron, forming a 6-coordinated species [42,43]. It is possible that, in extracted microsomes, some of the cytochrome P-450 is converted to the low spin form as a result of binding to 1-butanol. It is not likely that formation of low spin cytochrome P-450 is responsible for the kinetic changes observed. Addition of 1-butanol to unextracted microsomes at the concentration (~0.2 mM) calculated to be present in extracted microsomes had no effect on benzo[*a*]pyrene hydroxylase activity. Furthermore, extraction of microsomes with acetone alone had no effect on benzo[*a*]pyrene hydroxylase activity. Acetone also has a sterically accessible oxygen atom for binding to the ferric ion of cytochrome P-450 forming the 6-coordinated species and induces the modified Type II binding spectra. It should be noted that the benzo[*a*]pyrene hydroxylase assay is routinely carried out in the presence of acetone (0.3 M) used as a solvent for benzo[*a*]pyrene.

The formation of low spin cytochrome P-450 in extracted microsomes could explain the increased Δ absorption maximum in the hexobarbital- and benzphetamine-induced Type I binding spectra observed in extracted microsomes [7], since Kumaki *et al.* [43] have reported that the amount of cytochrome P-450 converted to the high spin form by exogenous Type I ligands is increased in microsomes containing greater amounts of low spin cytochrome P-450. In these same microsomal preparations, however, the phenacetin- and ethanol-induced modified Type II spectra and the aniline-induced Type II spectra Δ absorption maximum were also increased in extracted microsomes. Ebel *et al.* [44] and others [45–47] have shown that a limited amount (approximately 35 per cent) of the cytochrome P-450 in microsomes participates (measurably) in substrate binding, regardless of the concentration of substrate present. Extraction of microsomes with 1-butanol, therefore, appears to increase the amount of cytochrome P-450 which measurably binds to Type I, Type II and modified Type II exogenous ligands. This could be due either to the removal of endogenous ligands or to the removal of non-specific binding sites in the membrane.

Because of the presence of multiple forms of cytochrome P-450 with different substrate specificities in microsomes, the K_m values obtained in microsomes must be interpreted cautiously, since they represent the average K_m of the different forms. Nevertheless, the present studies show that components of the microsomal membrane play an important role in regulating benzo[*a*]pyrene hydroxylase activity in control microsomes and that the interactions between the membrane components and cytochrome P-450 are altered by induction with 3-MC. Benzo[*a*]pyrene has been shown to be metabolized by cytochrome P-450 to highly reactive diol-epoxides which are potent mutagens and carcinogens [48–50]. The low affinity of benzo[*a*]pyrene for cytochrome P-450 in control microsomes, whether due to the presence of endogenous substrates which are competitive inhibitors or to lipid-protein interactions,

may serve as a means of protecting the hepatocyte from the formation of these carcinogenic metabolites.

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