Phospholipid Requirement for Dimethylnitrosamine Demethylation by Hamster Hepatic Microsomal Cytochrome *P*-450 Enzyme System

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Extraction with butan-1-ol of freeze-dried microsomal fractions from livers of 3-methylcholanthrene-pre-treated hamsters removed about 90% of the total lipid content, but the lipid remaining proved sufficient for the cytochrome *P*-450 enzyme system to retain about 15–40% of its original catalytic activity for dimethylnitrosamine demethylation. Addition of butan-1-ol-extracted total phospholipid or phosphatidylcholine could not restore any activity, whereas the addition of the synthetic phospholipid dilauroyl phosphatidylcholine was able to restore almost complete activity. Synthetic dipalmitoyl or distearoyl phosphatidylcholine was ineffective in restoring the activity in this reconstituted system.

It has been demonstrated with solubilized and resolved mammalian liver microsomal mixed-function oxidase system that all three resolved fractions, cytochrome *P*-450, NADPH-cytochrome *P*-450 reductase and phosphatidylcholine, are required for oxidation of a variety of physiological and xenobiotic compounds (Lu *et al.*, 1969, 1973; Strobel *et al.*, 1970).

The chemical carcinogen dimethylnitrosamine is oxidatively demethylated by a mammalian liver microsomal cytochrome *P*-450 enzyme system (Czygan *et al.*, 1973). Such an oxidative process has been suggested to be an activation step in its carcinogenic process (Druckrey *et al.*, 1967; Magee & Barnes, 1967; Miller, 1970). In our previous reconstitution studies with rat and hamster liver microsomal fractions, we demonstrated that both cytochrome *P*-450 and NADPH-cytochrome *c* reductase fractions were required for optimum dimethylnitrosamine demethylation activity (Lotlikar *et al.*, 1975). Differences in demethylation activity with liver preparations from these two species appeared to be due to differences in specificity of their cytochrome *P*-450 fractions. Our inability to demonstrate any lipid requirement in those studies might have been due to incomplete removal of either endogenous phospholipid or Triton X-100 solubilizer.

The present paper now demonstrates a requirement for a phospholipid in oxidative de-alkylation of dimethylnitrosamine by butan-1-ol-extracted microsomal fractions from livers of 3-methylcholanthrene-pre-treated hamsters.

Materials and Methods

Chemicals used in the present study were from the following sources: dilauroyl phosphatidylcholine,

dipalmitoyl phosphatidylcholine, NADPH and horse heart cytochrome *c* (type III), Sigma Chemical Co., St. Louis, MO, U.S.A.; distearoyl phosphatidylcholine, Calbiochem, La Jolla, CA, U.S.A.; di[¹⁴C]methyl nitrosamine (sp. radioactivity 4.8 mCi/mmol), New England Nuclear Corp., Boston, MA, U.S.A.; dimethylnitrosamine and 3-methylcholanthrene, Eastman Kodak Co., Rochester, NY, U.S.A. All other chemicals were of reagent grade.

Adult male Syrian golden hamsters (100–150 g body wt.) were injected intraperitoneally with 3-methylcholanthrene (100 mg/kg body wt.) suspended in 1 ml of corn oil, 24 h before death. The pooled livers of 12–16 animals were homogenized in 3 vol. of ice-cold 0.25 M-sucrose and the microsomal fraction was isolated as described previously (Lotlikar *et al.*, 1974). After the surface of the microsomal pellet was washed three times with 0.25 M-sucrose and twice with water, it was suspended in water and freeze-dried. One portion of freeze-dried microsomal powder containing about 250 mg of protein was homogenized in 10 ml of 0.25 M-sucrose containing 10% (v/v) glycerol and was then sonicated (model W185 Sonifier Cell Disrupter; Heat Systems-Ultrasonics Inc., New York, NY, U.S.A.) twice at no. 6 speed for 15 s at 4°C. This microsomal fraction is designated as the control or unextracted group. Another portion of freeze-dried microsomal powder was extracted with butan-1-ol by a slightly modified procedure of Narasimhulu (1974) and Vore *et al.* (1974a). In brief, freeze-dried powder containing 500 mg of protein was extracted with 50 ml of cold anhydrous butan-1-ol (10 mg of protein/ml of butan-1-ol) by homogenization. The suspension was centrifuged at 35 000 g for 5 min at 4°C. The pellet was re-extracted with the same amount of butan-1-ol and re-centrifuged as above. The pellet was sus-

pended in acetone, filtered over a small Buchner funnel and was washed with 100ml of acetone. After the residue was dried in a desiccator under vacuum for 30min, it was homogenized in 20ml of 0.25M-sucrose containing 10% (v/v) glycerol and sonicated as described above. Both of these microsomal fractions (unextracted and extracted), when kept frozen at -15°C , were stable for at least 1–2 weeks.

The two butanol extracts of microsomal fractions were combined and the butanol was removed under reduced pressure. The residue was dissolved in a small amount of chloroform and the lipids were fractionated by column chromatography on silicic acid by a modified procedure of Strobel *et al.* (1970). After glycolipids and neutral lipids were eluted from the column with acetone, the phospholipid fraction was eluted from the column with methanol. The phospholipid fraction was further resolved by preparative t.l.c. on silica gel G plates in chloroform/methanol/water (65:25:4, by vol.) and the phospholipid bands were identified with the phosphomolybdate spray described by Dittmer & Lester (1964). The phosphatidylcholine band was eluted with methanol and kept under N_2 at -15°C . Extracted total lipids and phospholipids were also stored in methanol under N_2 at -15°C . Total lipids were also obtained from liver microsomal fractions by extraction with chloroform/methanol (1:1, v/v) by the method of Bligh & Dyer (1959). Phospholipids were also isolated and separated from this extract as described above.

Total phospholipid phosphorus present in microsomal fractions was determined by the method of Bartlett (1959). Cytochrome *P*-448 content and NADPH-cytochrome *c* reductase were assayed by the methods of Omura & Sato (1964) and Masters *et al.* (1971) respectively. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

$\text{Di}[^{14}\text{C}]$ methylnitrosamine (sp. radioactivity 4.8 mCi/mmol) was diluted with unlabelled dimethylnitrosamine to yield $\text{di}[^{14}\text{C}]$ methylnitrosamine with a sp. radioactivity of 0.1 mCi/mmol. Lipid fractions dissolved in methanol were dried under N_2 and residues were suspended in 0.5 M-potassium phosphate buffer, pH 6.5. Lipid suspensions were sonicated for 3×10 s intervals just before use. The complete incubation medium for dimethylnitrosamine demethylation contained 0.1 M-potassium phosphate buffer, pH 6.5, 2 mM-NADPH, 0.1 M-semicarbazide, 2 mM- $\text{di}[^{14}\text{C}]$ methylnitrosamine (sp. radioactivity 0.1 mCi/mmol), liver microsomal fraction containing 1 nmol of cytochrome *P*-448, 0.05 M-sucrose, 2% (v/v) glycerol and lipid fractions (as indicated) in a total volume of 1.0 ml. Samples, which contained all the indicated components except NADPH, were pre-incubated for 5 min at 37°C . The reaction was initiated by the addition of NADPH and the samples were then incubated for 30 min at 37°C . The reaction

was terminated by the addition of 1.5 ml of 50% (w/v) trichloroacetic acid. Blank samples were prepared by adding NADPH after the addition of trichloroacetic acid. Oxidative demethylation of dimethylnitrosamine was determined by measuring the formation of $[^{14}\text{C}]$ formaldehyde, which was precipitated as formaldemethone as described previously (Lotlikar *et al.*, 1975). Enzyme activity is expressed in terms of nmol of $[^{14}\text{C}]$ formaldehyde formed/30 min per nmol of cytochrome *P*-448.

Results and Discussion

Previous studies have shown that, in addition to cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase, a lipid factor, especially phosphatidylcholine, is required for oxidation of several physiological substrates such as steroids and fatty acids, and a variety of xenobiotics including drugs and some carcinogens (Lu *et al.*, 1969, 1973; Strobel *et al.*, 1970; Coon *et al.*, 1971; van der Hoeven & Coon, 1974). It was demonstrated that the active lipid, a phosphatidylcholine, neither caused spectral changes when added to cytochrome *P*-450 nor did it alter the binding of substrates such as laurate or benzphetamine to cytochrome *P*-450 (Lu *et al.*, 1969, 1970; Coon *et al.*, 1971). Phosphatidylcholine was found to be required for the enzymic reduction of cytochrome *P*-450 (Strobel *et al.*, 1970; Coon *et al.*, 1971). Several ionic and non-ionic detergents replace phosphatidylcholine in oxidation of various substrates in a liver microsomal reconstituted system (Lu & West, 1972).

In our earlier studies with rat and hamster liver reconstituted systems, exogenous lipid requirement for oxidation of carcinogens such as 2-acetamidofluorene and dimethylnitrosamine could not be demonstrated (Lotlikar *et al.*, 1974, 1975; Lotlikar & Zaleski, 1975). Similar results were also obtained for benzo[a]pyrene hydroxylation with reconstituted rat lung microsomal system (Jernstrom *et al.*, 1975). These investigators indicated that enough phospholipid might have been present as a contaminant in either cytochrome *P*-450 or reductase fraction or that Triton X-100 present in small amounts might have substituted for the phospholipid.

The present studies were designed to establish a phospholipid requirement in oxidative demethylation of dimethylnitrosamine by microsomal fractions from livers of 3-methylcholanthrene-pre-treated hamsters. It was found that, even though two butanol extractions removed almost 90 and 70% of total lipid and phosphatidylcholine from liver microsomal fraction respectively, NADPH-cytochrome *c* reductase activity was unaffected and cytochrome *P*-448 content was decreased only to about 70% of that of the control (unextracted) microsomal fraction. Under these conditions, oxidative demethylation activity was

Table 1. *Lipid requirement for dimethylnitrosamine demethylation by microsomal fractions from livers of 3-methylcholanthrene-pre-treated hamsters*

All details are described in the Materials and Methods section. Where indicated, synthetic lipid, either dilauroyl or distearoyl or dipalmitoyl phosphatidylcholine was added. Control (unextracted) microsomal fraction formed 40nmol of [14 C]formaldehyde/30min per nmol of cytochrome *P*-448.

Microsomal fraction	Phosphatidylcholine	[14 C]Formaldehyde formed (% of control)
Unextracted (control)	—	100
Unextracted	Dilauroyl (0.5 mg)	90
Unextracted	Dipalmitoyl (0.5 mg)	95
Unextracted	Distearoyl (0.5 mg)	98
Extracted	—	42
Extracted	Dilauroyl (0.1 mg)	69
Extracted	Dilauroyl (0.25 mg)	97
Extracted	Dilauroyl (0.5 mg)	105
Extracted	Dilauroyl (1.0 mg)	90
Extracted	Dipalmitoyl (0.5 mg)	42
Extracted	Distearoyl (0.5 mg)	41

Table 2. *Requirement of phospholipid for dimethylnitrosamine demethylation by microsomal fractions from livers of 3-methylcholanthrene-pre-treated hamsters*

All details are described in the Materials and Methods section. Where indicated, either synthetic phospholipid, dilauroyl phosphatidylcholine or butanol-extracted lipid from liver microsomal fraction was added. Control (unextracted) microsomal fraction formed 32nmol of [14 C]formaldehyde/30min per nmol of cytochrome *P*-448.

Microsomal fraction	Phospholipid	[14 C]Formaldehyde formed (% of control)
Unextracted control		100
Unextracted	Total lipid (extracted) (1.0mg)	90
Unextracted	Total phospholipid (extracted) (1.0mg)	93
Unextracted	Phosphatidylcholine (extracted) (0.5 mg)	101
Unextracted	Dilauroyl phosphatidylcholine (synthetic) (0.5 mg)	98
Extracted		14
Extracted	Total lipid (extracted) (0.5 or 1.0mg)	14
Extracted	Total phospholipid (extracted) (0.5 or 1.0mg)	15
Extracted	Phosphatidylcholine (extracted) (0.25 or 0.5 mg)	18
Extracted	Dilauroyl phosphatidylcholine (synthetic) (0.5 mg)	93

decreased to about 15–40% of control values (Tables 1 and 2). Such a decrease in activity could be restored completely in the presence of a synthetic phospholipid, dilauroyl phosphatidylcholine. However, the presence of other synthetic phospholipids such as distearoyl or dipalmitoyl phosphatidylcholine was ineffective in restoring the demethylation activity (Table 1). All of these synthetic phosphatidylcholine esters did not have any appreciable effect on the demethylation activity of unextracted liver microsomal fraction.

In contrast with complete restoration of demethylation activity in the presence of dilauroyl phosphatidylcholine, addition of either phosphatidylcholine or total phospholipid isolated from butanol extracts

did not increase the demethylation activity of extracted liver microsomal fraction (Table 2).

Our present data (Tables 1 and 2) do demonstrate a phospholipid, especially dilauroyl phosphatidylcholine, requirement for dimethylnitrosamine demethylation by microsomal fraction from livers of 3-methylcholanthrene-pre-treated hamsters. Similar results have also been obtained with liver microsomal preparations from control hamsters (P. D. Lotlikar, unpublished work). These present results are in agreement with those of Vore *et al.* (1974*a,b*); in their reports benzo[*a*]pyrene hydroxylation was studied with microsomal fractions from livers of 3-methylcholanthrene-pre-treated rats. However, in contrast with their data (Vore *et al.*, 1974*a,b*), in the present

studies we were unable to demonstrate restoration of activity with phosphatidylcholine fraction from either butanol extract or chloroform/methanol extract. In other studies, however, butanol-extracted phosphatidylcholine fraction was able to restore *N*- and *ring*-hydroxylation of 2-acetamidofluorene (P. D. Lotlikar & E. N. Dwyer, unpublished work). All of these results suggest that, in addition to being involved in enzymic reduction of cytochrome *P*-450, phosphatidylcholine might have another role, which might demonstrate substrate specificity in overall oxidation reactions.

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