Phospholipid Requirement for 2-Acetamidofluorene *N*- and Ring-Hydroxylation by Hamster Liver Microsomal Cytochrome *P*-450 Enzyme System

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A phospholipid requirement for 2-acetamidofluorene *N*- and ring-hydroxylation was investigated with partially delipidated microsomal fraction from livers of 3-methylcholanthrene-pretreated hamsters. Butan-1-ol extraction of microsomal fraction removed 90% of the total lipid content without any appreciable effect on microsomal proteins. Such extracted microsomal fractions had much lower capacity to *N*- and ring-hydroxylate 2-acetamidofluorene: 25 and 44% of control respectively. Addition of butan-1-ol-extracted total lipid restored both oxidations to some extent, whereas addition of phosphatidylcholine fraction restored both oxidations almost completely. Addition of synthetic phospholipid, dilauroyl phosphatidylcholine, restored both oxidations to a large extent, whereas synthetic dipalmitoyl or distearoyl phosphatidylcholine was ineffective in restoring these oxidations.

A requirement for phospholipid has been established for the oxidation of a variety of physiological and foreign compounds by the solubilized and reconstituted hepatic cytochrome *P*-450 enzyme system (Lu *et al.*, 1969, 1973; Strobel *et al.*, 1970).

Several studies have shown that *N*-hydroxylation and ring-hydroxylation are activation and inactivation steps respectively in the carcinogenesis by 2-acetamidofluorene and several other aromatic amides and amines (Miller, 1970; Weisburger & Weisburger, 1973). On the basis of CO-inhibition studies, it appears that both *N*- and ring hydroxylation of 2-acetamidofluorene are mediated via the cytochrome *P*-450 mixed-function oxidase system (Thorgeirsson *et al.*, 1973; Lotlikar & Zaleski, 1974; Gutmann & Bell, 1977). However, multiple forms of cytochrome *P*-450 have been suggested to be involved in *N*- and ring-hydroxylation of this carcinogenic aromatic amide because of differential inhibition of these oxidations by CO (Lotlikar & Zaleski, 1974). In hamsters, pretreatment with 3-methylcholanthrene produces a severalfold increase in hepatic microsomal *N*-hydroxylation without appreciably increasing ring-hydroxylation activity (Lotlikar *et al.*, 1967). In our previous reconstitution studies with both rat and hamster liver microsomal preparations, we showed that for 2-acetamidofluorene *N*- and ring-hydroxylation both cytochrome *P*-450 and NADPH-cytochrome *c* reductase fractions were required for optimum activity (Lotlikar *et al.*, 1974; Lotlikar & Zaleski, 1975). However, in these reconstitution

studies an absolute lipid requirement could not be easily demonstrated.

In the present study, we now demonstrate a phospholipid requirement for 2-acetamidofluorene *N*- and ring-hydroxylation by delipidated microsomal fractions from livers of 3-methylcholanthrene-pretreated hamsters.

Materials and Methods

Chemicals used in the present study were from the following sources: horse heart cytochrome *c* (type III), NADPH, dilauroyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, Sigma Chemical Co., St. Louis, MO, U.S.A.; 2-acetamidofluorene and 2-acetamido[9-¹⁴C]fluorene (sp. radioactivity 86 mCi/mmol), Schwarz-Mann Co., Orangeburg, NY, U.S.A.; distearoyl phosphatidylcholine, Calbiochem, La Jolla, CA, U.S.A.; 3-methylcholanthrene, Eastman Kodak Co., Rochester, NY, U.S.A. All other chemicals were of reagent grade.

A group of 12–16 adult male Syrian hamsters (100–150 g body wt.) was injected intraperitoneally with 3-methylcholanthrene (100 mg/kg body wt.), suspended in corn oil, 24 h before death. After preparation of liver microsomal fraction by the procedure described previously (Lotlikar *et al.*, 1974), the microsomal pellet was suspended in water and freeze-dried. One portion of the freeze-dried microsomal powder was homogenized in 0.25 M-sucrose containing 10%

(v/v) glycerol and was then sonicated for 2×15 s periods at 4°C . This microsomal fraction is designated as the control or unextracted group. Another portion of the freeze-dried microsomal powder was extracted with butan-1-ol by a slightly modified procedure of Narasimhulu (1974) and Vore *et al.* (1974a) as described previously (Lotlikar *et al.*, 1976). In brief, freeze-dried powder was extracted twice with cold anhydrous butan-1-ol by homogenization. The suspension was centrifuged at $35000g$ for 5 min at 4°C . The pellet was suspended in acetone and filtered. After the residue was dried in a desiccator under vacuum for 30 min, it was homogenized in 0.25M-sucrose containing 10% (v/v) glycerol and sonicated as above.

Total lipid was the residue obtained by removal of butanol under reduced pressure from butanol extracts of microsomal fraction. The phospholipid fraction from total lipids was obtained by a modified procedure of Strobel *et al.* (1970) using silicic acid column chromatography. 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 phospholipid phosphorus present in various microsomal fractions was determined by the method of Bartlett (1959).

The concentration of cytochrome *P*-448 was determined by the method of Omura & Sato (1964) from the CO-difference spectra of dithionite-reduced samples by using an extinction coefficient of $91 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ between 448 and 490 nm. The method of Masters *et al.* (1971) was used for the assay of NADPH-cytochrome *c* reductase activity. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Lipid fractions dissolved in methanol were dried under N_2 and were suspended in potassium phosphate buffer, pH 7.8. Lipid suspensions were sonicated for 3×10 s periods just before use. The complete incubation medium for 2-acetamidofluorene *N*- and ring-hydroxylation contained $50 \mu\text{mol}$ of potassium phosphate buffer, pH 7.8, $6 \mu\text{mol}$ of NADPH, $1.12 \mu\text{mol}$ of 2-acetamidofluorene containing $0.2 \mu\text{Ci}$ of 2-acetamido[9- ^{14}C]fluorene dissolved in 0.05 ml of methanol, $300 \mu\text{mol}$ of KF (to inhibit deacetylase activity), liver microsomal fraction containing 2.0 nmol of cytochrome *P*-448 and lipid fractions (as indicated) in a total volume of 3.0 ml. Duplicate samples were incubated in air for 20 min at 37°C . Extraction of hydroxylated metabolites of 2-acetamidofluorene from the incubation medium with diethyl ether, their separation by paper chromatography and radio-

activity measurements were as described previously (Lotlikar *et al.*, 1974).

Results and Discussion

Several reconstitution studies with hepatic microsomal enzyme systems have demonstrated that a lipid factor, especially phosphatidylcholine, is necessary in addition to cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase for oxidation of several foreign and some physiological compounds (Lu *et al.*, 1969, 1973; Strobel *et al.*, 1970; Coon *et al.*, 1971; vander Hoeven & Coon, 1974). In those studies a phosphatidylcholine requirement was shown to be necessary for the reduction of cytochrome *P*-450 (Strobel *et al.*, 1970; Coon *et al.*, 1971). In our previous reconstitution studies with rat and hamster liver microsomal fractions, an absolute lipid requirement for 2-acetamidofluorene oxidation could not be easily demonstrated (Lotlikar *et al.*, 1974; Lotlikar & Zaleski, 1975). This failure might have been due to incomplete removal of phospholipid or Triton X-100 from cytochrome *P*-450 fractions. Under such conditions, Triton X-100 might have substituted for the phospholipid in the carcinogen oxidation, as also reported by Lu & West (1972). Phospholipid requirement could not also be shown for benzo[*a*]pyrene hydroxylation with a reconstituted rat lung microsomal system (Jernstrom *et al.*, 1975).

In the present study the role of lipid in 2-acetamidofluorene oxidation was investigated by use of partially delipidated microsomal preparations. When freeze-dried microsomal powder was extracted with anhydrous butan-1-ol, there was an appreciable loss of lipid component without any significant loss of microsomal proteins. Thus the extracted microsomal fraction retained about 70% of its initial cytochrome *P*-448 content without any appreciable loss of its NADPH-cytochrome *c* reductase activity. However, these extracted microsomal fractions retained only 10 and 30% of their initial total lipid and phosphatidylcholine contents respectively. Under these conditions the extracted microsomal fraction had much lower capacity to *N*- and ring-hydroxylate 2-acetamidofluorene: 25 and 44% of control respectively (Table 1). Extracted total lipids at 1 mg/3 ml could restore these oxidations to some extent; however, higher concentration of total lipid (2 mg/3 ml) eliminated this increase. Phosphatidylcholine (extracted) at 1–2 mg/3 ml had an appreciable stimulatory effect on both types of oxidations. Under these conditions, synthetic phospholipid, dilauroyl phosphatidylcholine, at 0.5 mg/3 ml was most effective in restoring both oxidations. At higher concentrations (2–3 mg/3 ml) this phospholipid had inhibitory effect on both types of oxidations, preferentially on ring-hydroxylation, by extracted microsomal fractions. However, the presence of other synthetic phospholipids, such as

Table 1. *Phospholipid requirement for 2-acetamidofluorene hydroxylation by microsomal fractions from livers of 3-methylcholanthrene-pretreated hamsters*

All details are described in the Materials and Methods section. Where indicated, either synthetic phospholipid or butanol-extracted lipid from liver microsomal fraction was added (concentrations given as mg/3 ml reaction volume). Control (unextracted) microsomal fractions formed 16 ± 1.5 and 31 ± 2.9 nmol of *N*- and ring-hydroxylated 2-acetamidofluorene/20 min per nmol of cytochrome *P*-448 respectively. Results are averages of three analyses and are expressed as percentages of the control with unextracted microsomal fraction; experimental variations were less than 10% of the average of three analyses.

Microsomal fraction	Lipid	2-Acetamidofluorene hydroxylation (% of control)	
		<i>N</i> -Hydroxylation	Ring-hydroxylation
Unextracted	Control	100	100
Unextracted	Dilauroyl phosphatidylcholine (synthetic) (0.5 mg)	88	96
Unextracted	Dilauroyl phosphatidylcholine (synthetic) (1.0 mg)	52	60
Unextracted	Dilauroyl phosphatidylcholine (synthetic) (2.0 mg)	42	42
Extracted	Control	25	44
Extracted	Total lipid (extracted) (1.0 mg)	50	77
Extracted	Total lipid (extracted) (2.0 mg)	32	47
Extracted	Total phospholipid (extracted) (1.0 mg)	38	45
Extracted	Total phospholipid (extracted) (2.0 mg)	44	46
Extracted	Phosphatidylcholine (extracted) (0.5 mg)	40	51
Extracted	Phosphatidylcholine (extracted) (1.0 mg)	71	73
Extracted	Phosphatidylcholine (extracted) (2.0 mg)	90	85
Extracted	Dilauroyl phosphatidylcholine (synthetic) (0.5 mg)	90	75
Extracted	Dilauroyl phosphatidylcholine (synthetic) (1.0 mg)	90	59
Extracted	Dilauroyl phosphatidylcholine (synthetic) (2.0 mg)	65	30
Extracted	Dilauroyl phosphatidylcholine (synthetic) (3.0 mg)	61	25
Extracted	Dipalmitoyl phosphatidylcholine (synthetic) (1.0 mg)	22	40
Extracted	Distearoyl phosphatidylcholine (synthetic) (1.0 mg)	26	47

distearoyl or dipalmitoyl phosphatidylcholine at 1 mg/3 ml, was ineffective in restoring either the *N*- or ring-hydroxylation. In contrast with stimulatory effect on extracted microsomal fraction, synthetic dilauroyl phosphatidylcholine at 1 mg/3 ml and higher concentrations had inhibitory effect on both *N*- and ring-hydroxylation by unextracted microsomal fraction (Table 1).

Our present data demonstrate a requirement for phospholipid, especially dilauroyl phosphatidylcholine, for 2-acetamidofluorene *N*- and ring-hydroxylation by partially delipidated microsomal fraction from livers of 3-methylcholanthrene-pretreated hamsters. These results are in agreement with other results on benzo[*a*]pyrene hydroxylation (Vore *et al.*, 1974*a,b*) and dimethylnitrosamine demethylation (Lotlikar *et al.*, 1976). The present results are also in agreement with other studies (Vore *et al.*, 1974*a,b*) where the phosphatidylcholine fraction isolated from butanol extract was effective in restoring carcinogen oxidation. However, such a phospholipid fraction was ineffective in restoring dimethylnitrosamine demethylation (Lotlikar *et al.*, 1976). These subtle differences in lipid-dependency for oxidation of various substrates by cytochrome *P*-450 enzyme systems cannot be explained at the present time and merit further investigations.

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