ORGANIC SOLVENT EXTRACTION OF LIVER MICROSOMAL LIPID.

I. The Requirement of Lipid for 3,4-Benzpyrene Hydroxylase.

Mary Vore, James G. Hamilton, and Anthony Y. H. Lu

Department of Biochemistry and Drug Metabolism
Hoffmann-La Roche Inc.
Nutley, New Jersey 07110

Received December 28,1973

SUMMARY: Extraction of lyophilized microsomes from 3-methylcholanthrene treated rats with n-butanol and acetone removed all of the neutral lipid, 80% of the phosphatidylcholine and phosphatidylethanolamine, and 75% of the total phospholipid phosphorus. Recoveries of cytochrome P-448 and NADPH-cytochrome c reductase were 70-85%. The 3,4-benzpyrene hydroxylase activity was reduced to 40-60% of control and could be restored to full activity by the addition of liposomes of total microsomal lipid, or synthetic phosphatidylcholine. These results indicate that lipid is an essential component of the microsomal hydroxylation system.

Several studies in recent years have demonstrated a lipid dependency of the mixed-function oxidase system of liver microsomes. The reconstituted system of Lu et al requires phosphatidylcholine for maximal activity (1-3). In addition, treatment of microsomes with phospholipase C or iso-octane, which results in removal of phospholipid, decreases the metabolism and binding of type I compounds (4-6). Some of these data have been challenged recently by Cater et al (7) who have found that deoxycholate inhibits the metabolism of type I compounds and that phosphatidylcholine can relieve this inhibition. Since in the resolved system the cytochrome P-450 and reductase were fractionated in the presence of deoxycholate, the role of lipid could be that of relieving the inhibition caused by the detergent.

In order to understand the mechanism of hydroxylation, it is necessary to establish if lipid is an essential component of the mixed-function oxidase system. Therefore, we have developed a method for removing lipid without the use of detergent or phospholipase C treatment. This study reports the removal of lipid with organic solvent, an accompanying decrease in hydroxylation activity, and a restoration of that activity by the addition of either liposomes prepared from total microsomal lipid, or synthetic phosphatidylcholine.

METHODS: Livers from 3-methylcholanthrene treated (25 mg/kg/day, 3 days) ıle, Long-Evans rats (180-200 g) were homogenized in 4 volumes of 1.15% KCl. The icrosomal pellet was washed in 1.15% KCl and finally suspended in distilled water ? x liver weight) and lyophilized. The evacuated flasks were flushed with Argon and :ored in a desiccator at -20°C for a maximum of one week.

Extraction of lipid from microsomes: In a typical experiment, 250 mg of /ophilized microsomes (containing 120 mg protein) were homogenized with 25 ml of -butanol (Matheson, Coleman & Bell, pesticidequality, East Rutherford, New Jersey) ıd centrifuged at 35,000 x g for 5 minutes. The pellet was rinsed twice with 25 ml f acetone (Matheson, Coleman & Bell,spectroquality), and the final acetone suspension iltered in a Buchner funnel. The fine, cream-colored powder was dried under N_2 and ıen placed in a desiccator under vacuum at 4°C for 30 minutes. The centrifuge and ll solvents were kept at -20°C, and the entire procedure was carried out at 4°C. The rocedure must be performed under strictly anhydrous conditions for minimal loss of ytochrome P-448 and NADPH-cytochrome c reductase. The dried powder was homogenized ı 60 ml of 0.1 M potassium phosphate buffer, pH 7.7, and sonicated at 4°C for five 5- econd intervals. Control microsomes were prepared by homogenizing 130 mg lyophilized icrosomes (containing 60 mg of protein) in 40 ml of the buffer and sonicated as escribed above for a total of 15 seconds. Sonication served to keep the microsomes ı suspension and was found to be essential for a consistent reactivation of the ydroxylase activity. Total phospholipid phosphorus present in control and extracted icrosomes was determined by the method of Bartlett (8).

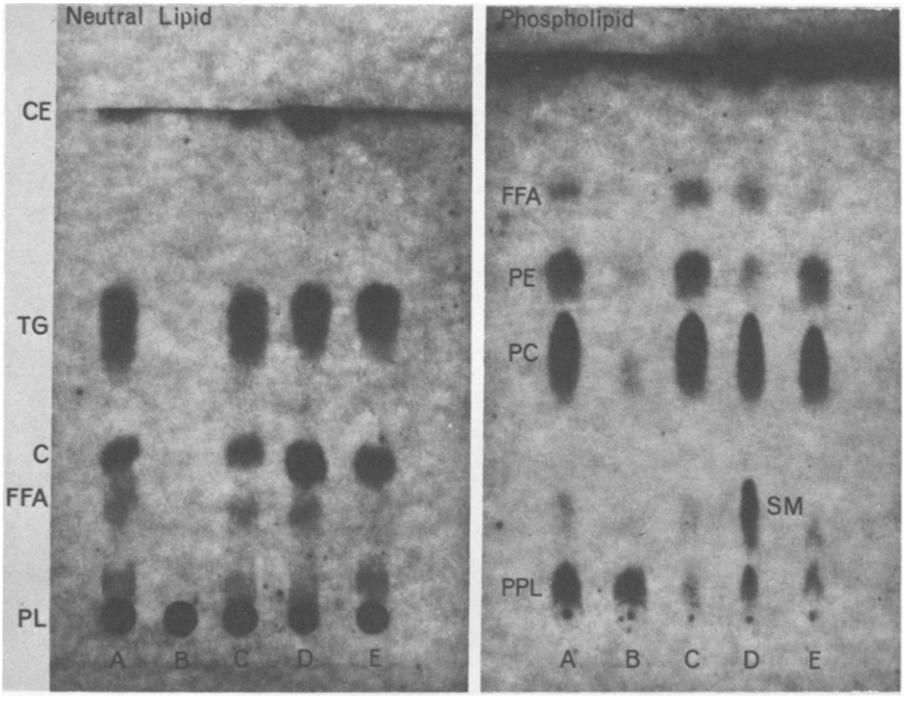
Preparation of liposomes: Total microsomal lipid was extracted from 200 mg f lyophilized microsomes by the method of Bligh and Dyer (9). To prepare liposomes, ıe organic solvent was evaporated under a stream of nitrogen and the dry lipid sus- ınded in 10 ml of 0.02 M Tris buffer pH 8.1 containing 10^{-3} M EDTA. The suspension ıs sonicated for 5 minutes at 4°C under a stream of nitrogen and was then centrifuged : 140,000 x g for 60 minutes to remove any undispersed lipid. The resulting sus- ınsion contained 4.3 mg/ml of total lipid (determined gravimetrically) and could be tored for one week at 4° under nitrogen.

Analysis of lipid extracts: The lipid content of the control and extracted microsomes and of the n-butanol extract were analyzed by ^{14}C-labelling experiments and by thin layer chromatography (TLC). The details of the methodology are described in the legend.

Other assays: Cytochrome P-448 (10), NADPH-cytochrome c reductase (11), protein (12), and 3,4-benzpyrene hydroxylation (13,14) were assayed by established procedures. In experiments in which liposomes of total lipid were used to restore activity, both control and extracted microsomes were pre-incubated with appropriate amounts of lipid for 45 minutes at 37° C. Loss of cytochrome P-448 due to the preincubation was less than 10%.

RESULTS AND DISCUSSION: Extraction of tissues with n-butanol has been used previously as a method for removing lipid (15-18). Figure 1 shows the thin layer chromatograms of control and extracted microsomes and of the n-butanol extract. Essentially all of the neutral lipid was removed by the n-butanol extract. Preliminar experiments designed to determine the effectiveness of n-butanol in removing lipid showed that approximately 90% of the phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) were removed by two n-butanol extracts, but as many as five extractions only partially removed the more polar phospholipids, probably consisting of phosphatidylinositol (PI) and phosphatidylserine. Although multiple n-butanol extract removed more phospholipids, the reactivation of 3,4-benzpyrene hydroxylase activity by the addition of microsomal lipid was highly variable. Therefore, to obtain consistent results, all studies reported here were done after one n-butanol and two acetone extractions. As shown in Table I, 80% of the ^{14}C-PC and ^{14}C-PE, 20% of the ^{14}C-PI and 70% of the total phospholipid phosphorus were removed by one n-butanol and two acetone extractions.

Cytochrome P-450 and NADPH-cytochrome c reductase in microsomal suspensions have been shown to be extremely labile to organic solvent extractions (17,18). However, these two enzymes were remarkably stable when lyophilized microsomes were extracted. The recovery of cytochrome P-448 and the reductase ranged from 70-85% (Table I).



e 1. Chromatograms of the neutral lipid (left) and phospholipids (right) present in control and extracted microsomes and in the n-butanol extract. Lyophilized microsomes (100 mg) were extracted with n-butanol (10 ml) and rinsed with acetone (2 x 10 ml). Chloroform: methanol (2:1) extracts of both control (A) and extracted (B) microsomes and a comparable aliquot of the n-butanol extract (C) were spotted on silica gel-impregnated glass filter paper (Gilman Instrument Co.) Serum lipid (D) and egg yolk lipid (E) were used as standards. Neutral lipids were chromatographed in an iso-octane: isopropyl acetate (50:1) solvent system; phospholipids in a chloroform: methanol:7N ammonia (200:25:3) solvent system. Chromatograms were visualized by spraying with H_2SO_4 and charring. CE = cholesterol esters; TG = triglycerides, C = cholesterol; FFA = free fatty acids; PL = phospholipids; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; PPL = polar phospholipids.

The enzymatic activity of microsomes after one n-butanol extraction and tone washings, as measured by the ability to hydroxylate 3,4-benzpyrene, was deased to 40-60% of the control activity (Figure 2). Activity is expressed as les product formed per 5 minutes per nmole of P-448 to correct for the loss of 48. Both total lipid liposomes and synthetic phosphatidylcholine (a mixture of o- and dilauroyl glyceryl-3-phosphorylcholine, generously furnished by Dr. M. J. Coon) tored full activity to the extracted microsomes. In the case of total lipid lipoes, it was necessary to preincubate the lipid with the microsomes for 45 minutes

Table I

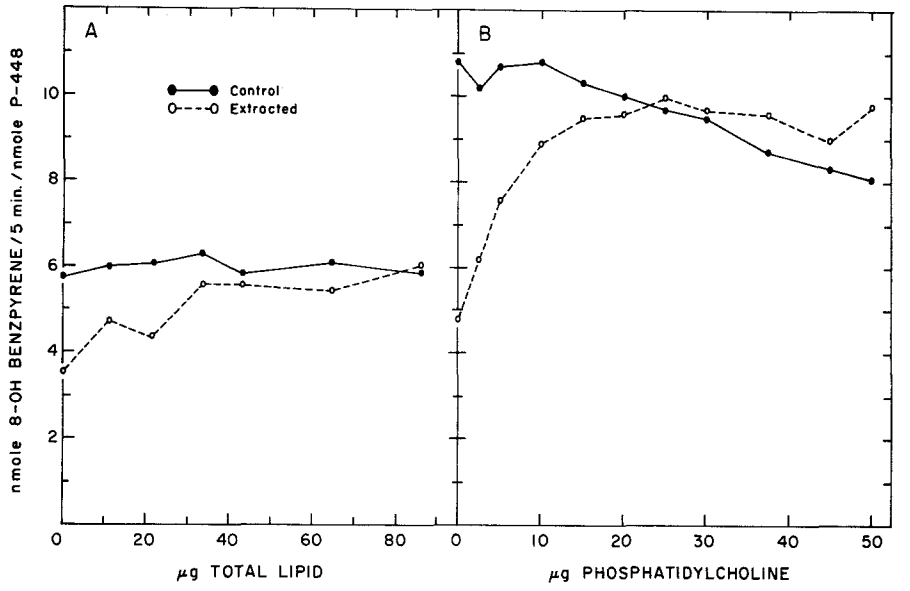
Effect of One n-Butanol and Two Acetone Extractions on Various Components of Microsomes

Components	Control	Extracted	% Remaining	Number of Experiments
Cytochrome P-448 (nmoles)	167	138	75 [70-85]	8
Reductase (nmoles cytochrome c reduced/minute)	17,970	14,675	80 [70-90]	5
Protein (mg)	120	120	100	3
^{14}C-Phosphatidylcholine (μCi)	.108	.023	20	2
^{14}C-Phosphatidylethanolamine (μCi)	.590	.100	18	2
^{14}C-Phosphatidylinositol (μCi)	.028	.022	80	2
Total phospholipid phosphorus (mg)	2.16	.60	28	3

Lyophilized microsomes (250 mg) were suspended in 0.1 M potassium phosphate buffer pH 7.7 (Control) or extracted once with n-butanol and twice with acetone and then suspended in the buffer (Extracted). Microsomal components were measured as described in Methods. The range of recoveries for cytochrome P-448 and reductase is given in brackets. In experiments designed to quantitate the extraction of specific phospholipids, rats were injected i.p. with either 12 μCi ^{14}C-choline (6.2 mCi/mmol), 8.7 μCi ^{14}C-ethanolamine (2.0 mCi/mmol) or 4 μCi ^{14}C-myo-inositol (2.1 mCi/mmol) (New England Nuclear) one hour prior to sacrifice. Aliquots of the Control and Extracted microsomes and of the n-butanol extract were then assayed for ^{14}C.

at 37°C to obtain full reactivation consistently. No preincubation was required for full reactivation with the synthetic phosphatidylcholine, however. Neither deoxycholate nor cholate, at concentrations which did not inhibit hydroxylation, restored activity to the extracted microsomes.

These data show conclusively that lipid is required for full 3,4-benzpyrene hydroxylase activity and that the lipid requirement is unrelated to either the use of detergent or phospholipase C.



re 2. Reactivation of 3,4-benzpyrene hydroxylase activity of n-butanol and acetone extracted microsomes by the addition of total microsomal lipid (A) or synthetic phosphatidylcholine (B). Total microsomal lipid was preincubated with control and extracted microsomes for 45 minutes at 37°C to give consistent reactivation. The reaction mixture (1 ml) contained 100 µmoles potassium phosphate buffer pH 7.3, 3 µmoles $MgCl_2$, 0.4 µmoles NADPH, 80 nmoles 3,4-benzpyrene (in 40 µl acetone), 0.10 mg protein, and the indicated amount of total lipid or phosphatidylcholine. The reaction mixture was incubated at 37°C for 5 minutes and 8-hydroxy-3,4-benzpyrene determined (13,14).

Acknowledgments: We thank Drs. Allan Conney and R. Kuntzman for helpful suggestions and Mrs. Cathy Chvasta and Mrs. P. Althoff for their assistance in preparing the manuscript.

REFERENCES

1. Lu, A. Y. H., and Coon, M. J., J. Biol. Chem. 243, 1331 (1968).
2. Lu, A. Y. H., Strobel, H. W., and Coon, M. J., Biochem. Biophys. Res. Comm. 36, 545 (1969).
3. Strobel, H. W., Lu, A. Y. H., Heidema, J., and Coon, M. J., J. Biol. Chem. 245, 4851 (1970).
4. Chaplin, M. D., and Mannering, G. J., Mol. Pharmacol. 6, 631 (1970).
5. Eling, T. E., and DiAugustine, R. P., Biochem. J. 123, 539 (1971).
6. Leibman, K. C., and Estabrook, R. W., Mol. Pharmacol. 7, 26 (1971).
7. Cater, B. R., Walkden, V., Hallinan, T., Biochem. J. 127, 37P (1972).

8. Bartlett, G. R., J. Biol. Chem. 234, 466, (1959).
9. Bligh, E. G., and Dyer, W. J., Can. J. Biochem. Physiol. 37, 911 (1959).
10. Omura, T., and Sato, R., J. Biol. Chem. 239, 2370 (1964).
11. Phillips, A. H., and Langdon, R. G., J. Biol. Chem. 237, 2652 (1962).
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
13. Nebert, D. W., and Gelboin, H. V., J. Biol. Chem. 243, 6242 (1968).
14. Lu, A. Y. H., Kuntzman, R., West, S., Jacobson, M., and Conney, A. H., J. Biol. Chem. 247, 1727 (1972).
15. Morton, R. K., Nature 166, 1092 (1950).
16. Schleyer, H., Cooper, D. Y., and Rosenthal, O., J. Biol. Chem. 247, 6103 (1972).
17. Bar-Tana, J., Rose, G., and Shapiro, B., Biochem. J. 122, 353 (1971).
18. Beaufay, H., and de Duve, C., Bull. Soc. Chim. Biol. 36, 1551 (1954).
19. Imai, Y., and Sato, R., European J. of Biochem. 1, 419 (1967).
20. Williams, C. H., and Kamin, H., J. Biol. Chem. 237, 587 (1962).