RECONSTITUTED LIVER MICROSOMAL ENZYME SYSTEM THAT HYDROXYLATES DRUGS, OTHER FOREIGN COMPOUNDS AND ENDOGENOUS SUBSTRATES.

VII. Stimulation of Benzphetamine N-Demethylation by Lipid and Detergent.

Anthony Y. H. Lu, Wayne Levin, and Ronald Kuntzman

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

Received July 16,1974

SUMMARY: The resolved liver microsomal hydroxylation system required lipid for benzphetamine N-demethylation. Certain nonionic detergents, such as Emulgen 911, Triton N-101, and Triton X-100, at appropriate concentrations could substitute for lipid. These results suggest that lipid and detergent activate the cytochrome P-450-containing hydroxylation system by a similar mechanism, probably by enhancing the interaction between cytochrome P-450 and NADPH-cytochrome c reductase.

In a series of studies, Coon and coworkers have shown that the liver microsomal hydroxylation enzyme system consists of two protein components -- cytochrome P-450 and NADPH-cytochrome c reductase -- and a lipid factor -- identified as phosphatidylcholine (1-3). In these and subsequent studies, both cytochrome P-450 and NADPH-cytochrome c reductase were solubilized and fractionated in the presence of the ionic detergents, cholate or deoxycholate (4,5). Although the two protein components were contaminated with phospholipids, this reconstituted hydroxylation system still required the lipid fraction for the optimal metabolism of a variety of substrates such as drugs, fatty acids, steroids and carcinogens (4,6). The crude lipid fraction could be replaced by synthetic phosphatidylcholine (3). On the other hand, deoxycholate or cholate was rather ineffective.

Recently, nonionic detergents such as Emulgen 911, Triton N-101, and Renex 690 have been used for the purification of cytochrome P-450 and NADPH-cytochrome c reductase (7-11). The use of both ionic and nonionic detergents at different stages in the isolation procedure has made it

possible to eliminate cross contamination, to remove both neutral and phospholipids, and to substantially purify cytochrome P-450 and NADPH-cytochrome c reductase. In the course of our studies, we observed that some nonionic detergents could replace the lipid and function in its place in the reconstituted hydroxylation system. In this paper, we report on the ability of various detergents to support benzphetamine N-demethylation in combination with cytochrome P-450 and NADPH-cytochrome c reductase.

METHODS: Cytochrome P-450 from liver microsomes of phenobarbital-treated rats (male, Long-Evans, 50-60 g) was solubilized with sodium cholate and fractionated with ammonium sulfate and calcium phosphate gel (12). This preparation --- termed Step III P-450 (5) -- had a specific content of 5-6 nmoles of cytochrome P-450 per mg protein. Since the purpose of this study was to assess the effectiveness of various nonionic detergents in supporting hydroxylation by the reconstituted hydroxylation system, Step III P-450 was not further purified. Further purification in the presence of the nonionic detergent Emulgen 911 would have resulted in a preparation with a specific content of 10 (8). However, Emulgen 911 cannot be removed completely from this preparation, and it would have contained approximately 0.05 mg Emulgen 911 per nmole of cytochrome P-450. NADPH-cytochrome c reductase was solubilized from the liver microsomes of phenobarbital-treated rats with Triton N-101 (9) and chromatographed on a DEAE-cellulose column in the presence of Triton N-101. The Triton N-101 in the sample was removed by binding the reductase fraction to a second DEAE-cellulose column followed by repeated washings of the column. The reductase fraction was eluted in the presence of deoxycholate, concentrated, and passed through a Sephadex G-25 column to remove deoxycholate. The final reductase preparation had a specific activity of 3870 units (1 unit=1.0 nmole cytochrome c reduced/min.) per mg of protein. The crude lipid fraction was isolated from solubilized microsomes on a DEAE-cellulose column and contained deoxycholate, neutral and phospholipids (2,3). Benzphetamine N-demethylation

was assayed by following the rate of NADPH oxidation, as previously described (4). The activity was routinely corrected for the rate of NADPH oxidation in the absence of benzphetamine. Previous studies have established that with the reconstituted system, the rate of benzphetamine-dependent NADPH oxidation is equal to the rate of formaldehyde formation (6).

RESULTS: The rate of NADPH-dependent benzphetamine N-demethylation was very low when the reaction mixture contained only cytochrome P-450 and NADPH-cytochrome c reductase; maximal activity required the presence of lipid (Table I). Figure 1 and Table I show that in addition to the lipid fraction

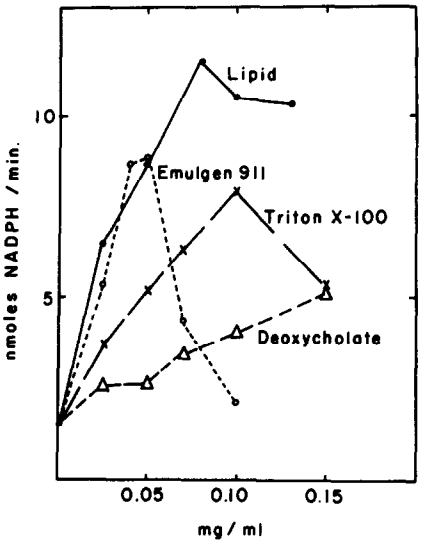

Figure 1. Stimulation of benzphetamine N-demethylation by lipid and detergent at various concentrations. Assay conditions were similar to those described in Table I.

---

or synthetic phosphatidylcholine (dilauroyl), a number of nonionic detergents could also stimulate the rate of benzphetamine N-demethylation. Emulgen 911, Triton N-101, Triton N-111, Triton X-100 were among the most effective. This stimulation by nonionic detergents could also be observed when more purified cytochrome P-450 (specific content, 9-11 nmoles/mg, reference 8) was used.

TABLE I

Stimulation of Benzphetamine N-Demethylation by Lipids and Detergents

Additions	Concentration for maximal activation (mg/ml)	% Activity
None	--	18
Crude lipid	0.10	100
Phosphatidylcholine, dilauroyl[a]	0.05	86
Emulgen 911[b]	0.04	81
Triton X-114[c]	0.05	48
Triton N-101[c]	0.04	75
Triton X-67[c]	0.05	48
Triton N-111[c]	0.05	70
Triton X-100[c]	0.10	73
T-DET A026[d]	0.05	27
Saponic 218[e]	0.03	37
Deoxycholate[f]	0.15	49
Cholate[g]	1.0	48

The reaction mixture, in a final volume of 1.0 ml, contained 100 μmoles of potassium phosphate buffer (pH 7.4), 0.1 μmole NADPH, 0.3 nmole P-450 (0.05 mg protein), 248 units NADPH-cytochrome c reductase (0.06 mg protein), 1 mM benzphetamine, and the indicated amounts of lipid or detergent. The rate of NADPH oxidation (nmoles/min. at 37°) was 10.8 in the presence of crude lipid and 1.94 in the absence of crude lipid after correction for the rate in the absence of benzphetamine. The initial rate of NADPH oxidation was determined at a series of lipid or detergent concentrations; the values shown in the Table are those which were obtained at the concentrations found to be optimal. [a]Supplied by Drs. M. J. Coon and H. E. Radtke; [b]from Kao-Atlas; [c]from Rohm and Haas; [d]from Thompson-Hayward Chemical Co.; [e]from Alcolac; [f]from Schwartz-Mann; [g]from Sigma.

In most cases, the concentration of these nonionic detergents which gave maximal stimulation fell into a very narrow range; higher concentrations often strongly

inhibited the reaction. Ionic detergents such as cholate and deoxycholate were much less effective. In the absence of benzphetamine, detergents did not enhance the endogenous NADPH oxidation.

When the lipid fraction was used, the order of addition of various components to the reaction mixture was important. Maximal activity was obtained when the lipid fraction was first mixed with cytochrome P-450 and NADPH-cytochrome c reductase; any other combination greatly decreased the rate of reaction (Table II). However, with Emulgen 911 or synthetic

TABLE II

Effect of Order of Addition of Lipid or Detergent on NADPH-dependent Benzphetamine N-demethylation

Order of Addition	nmoles NADPH/min.
A. With crude lipid	
Lipid + reductase + P-450 + buffer and cofactor	10.8
Reductase + P-450 + buffer and cofactor + lipid	5.5
Lipid + buffer and cofactor + reductase + P-450	5.5
B. With Emulgen 911	
Emulgen + reductase + P-450 + buffer and cofactor	8.2
Reductase + P-450 + buffer and cofactor + Emulgen	7.4
Emulgen + buffer and cofactor + reductase + P-450	7.4
C. With synthetic dilauroyl phosphatidylcholine (PC)	
PC + reductase + P-450 + buffer and cofactor	9.1
Reductase + P-450 + buffer and cofactor + PC	8.7
PC + buffer and cofactor + reductase + P-450	8.7

Assay conditions were similar to those described in Table I.

phosphatidylcholine, the order of addition was not a critical factor. The reason for these differences is not clear.

DISCUSSION: The results presented in this paper indicate that the lipid requirement for the liver microsomal hydroxylation system is not as specific as originally thought (1-4). Thus, like many other lipid-requiring enzyme systems (13,14), certain detergents can substitute for lipid. Recently, Narasimhulu (15) has reported that both lipid and nonionic detergent (such as Triton X-114) can reactivate the steroid C_{21}-hydroxylase (a cytochrome P-450-containing system) in lipid-depleted adrenal microsomes. The lack of specificity for the chemical structure of the lipid factor might indicate that lipid activates the cytochrome P-450-containing system by some physical rather than a specific chemical interaction. The requirement of lipid for the NADPH-dependent reduction of cytochrome P-450 (3) would thus suggest that phospholipid and detergent facilitate the interaction between NADPH-cytochrome c reductase and cytochrome P-450 in some unknown physical manner.

The fact that certain detergents can replace the lipid does not necessarily contradict the involvement of lipid in microsomal hydroxylation. Vore et al (16) have recently shown that the removal of microsomal lipid by organic solvent extraction results in a decrease in 3,4-benzpyrene hydroxylase activity; but activity can be fully restored by the addition of a total lipid extract or synthetic phosphatidylcholine to the lipid-depleted microsomes. Narasimhulu (15) has obtained similar results from lipid-depleted adrenal microsomes. These results indicate that the microsomal hydroxylation system is phospholipid dependent. Thus, in order to show a lipid requirement in a detergent-resolved hydroxylation system, one not only has to remove phospholipid from cytochrome P-450 and NADPH-cytochrome c reductase, but also must remove or minimize the nonionic detergent content in these enzyme preparations. It is also important to point out that high concentrations of nonionic detergent in a cytochrome P-450 or reductase preparation are extremely inhibitory to the overall hydroxylation reaction.

Acknowledgments: We thank Mrs. Cathy Chvasta for her assistance in preparing the manuscript.

REFERENCES

1. Lu, A. Y. H., and Coon, M. J. (1968) J. Biol. Chem. 243, 1331-1332.
2. Lu, A. Y. H., Junk, K. W., and Coon, M. J. (1969) J. Biol. Chem. 244, 3714-3721.
3. Strobel, H. W., Lu, A. Y. H., Heidema, J., and Coon, M. J. (1970) J. Biol. Chem. 245, 4851-4854.
4. Lu, A. Y. H., Kuntzman, R., West, S., Jacobson, M., and Conney, A. H. (1972) J. Biol. Chem. 247, 1727-1734.
5. Levin, W., Lu, A. Y. H., Ryan, D., West, S., Kuntzman, R., and Conney, A. H. (1972) Arch. Biochem. Biophys. 153, 543-553.
6. Lu, A. Y. H., Strobel, H. W., and Coon, M. J. (1970) Mol. Pharmacol. 6, 213-220.
7. Sato, R., Satake, H., and Imai, Y. (1973) Drug Metabolism and Disposition 1, 6-13.
8. Levin, W., Ryan, D., West, S. B., and Lu, A. Y. H. (1974) J. Biol. Chem. 249, 1747-1754.
9. Fujita, T., Shoeman, D. W., and Mannering, G. J. (1973) J. Biol. Chem. 248, 2192-2201.
10. Iyanagi, T., and Mason, H. S. (1973) Biochemistry 12, 2297-2308.
11. van der Hoeven, T. A., and Coon, M. J. (1974) J. Biol. Chem. 249, in press.
12. Lu, A. Y. H., and Levin, W. (1972) Biochem. Biophys. Res. Commun. 46, 1334-1339.
13. Martonosi, A., Donley, J., and Halpin, R. A. (1968) J. Biol. Chem. 243, 61-70.
14. Chuang, T. F., and Crane, F. L. (1973) Bioenergetics 4, 563-578.
15. Narasimhulu, S., The Second Philadelphia Conference on Heme Protein P-450, Philadelphia, 1974.
16. Vore, M., Hamilton, J. G., and Lu, A. Y. H. (1974) Biochem. Biophys. Res. Commun. 56, 1038-1044.