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Role of Phospholipids in the Hepatic Microsomal Drug-Metabolizing System

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SUMMARY

Cytochrome P-450, the terminal oxidase that functions in the oxidation of many drugs and other foreign compounds, is thought to depend in large degree for its physical and biochemical properties on its association with microsomal phospholipids. Drugs have been classified into two groups, depending upon whether they form a type I or a type II difference spectrum when they combine with cytochrome P-450. The type I binding site is thought to be located in an undetermined hydrophobic region of cytochrome P-450 protein or in lipids of the microsomal membrane, whereas the type II binding site is thought to be associated with the CO-binding site of the hemoprotein. Phospholipase C (EC 3.1.4.3), which specifically hydrolyzes phosphatidylcholines and phosphatidylethanolamines to corresponding phosphoryl products, was used in the current studies in an attempt to evaluate the role of phospholipids in the microsomal drug-metabolizing system. Untreated and phospholipase C-treated microsomes from the livers of rats were used to study the oxidation and binding of two type I drugs, ethylmorphine and hexobarbital, and the type II compound, aniline. Treatment of microsomes with phospholipase C destroyed the type I binding site, but microsomes lost only about 40% of their ability to oxidize ethylmorphine and hexobarbital. The loss of aniline oxidation was only about 15%, and the binding of aniline to hemoprotein was increased significantly. Phospholipase C treatment caused about a 20% conversion of cytochrome P-450 to cytochrome P-420, which remained with the microsomes. These studies provide further evidence that the type I and type II binding sites differ and that the type I binding site is associated with membrane phospholipids. The studies also show that type I binding is not required for the oxidation of type I compounds by microsomal enzymes.

INTRODUCTION

The physical and biochemical properties of cytochrome P-450, the terminal oxidase which functions in the hepatic microsomal system responsible for the metabolism of many drugs and other xenobiotics, are thought to depend to a large degree on associations of the hemoprotein with lipid components of the microsomal membrane (1–4). This view is based partly on the observation that agents which attack phospholipids, such as lipases contained in heated snake venom or crude pancreatic extracts, also convert the biochemically active cytochrome P-450 to the biochemically inactive cytochrome P-420.

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The crude lipase preparations employed to convert cytochrome P-450 to cytochrome P-420 contained a variety of lipid-splitting as well as proteolytic enzymes and therefore provided little information as to the nature of the association between cytochrome P-450 and microsomal phospholipid. The current studies have employed phospholipase C in an attempt to evaluate more specifically the role of phospholipids in the microsomal drug-metabolizing system. Phospholipase C (EC 3.1.4.3) was selected because of its availability in purified form and because of its established specificity in the hydrolysis of phosphatidylcholines and phosphatidylethanolamines to their corresponding α,β-diglycerides and the respective phosphoryl compounds. The specificities of phospholipases make them useful tools for the selective degradation of membrane structures (5–7). These enzymes may be used to alter or eliminate specific groups of phospholipids attached to biological membranes. Phospholipase C has been used to demonstrate a phospholipid requirement for ATPase activity and Ca^{++} transport in the sarcoplasmic reticulum (8), for the transport of Rb^{+} by kidney cortex tubules (9), and for microsomal NADH–cytochrome *c* reductase (10, 11) and glucose 6-phosphatase activities (11, 12).

Remmer and co-workers (13) and Imai and Sato (14) showed that drugs can be classified into two types, I and II,[2] in accordance with the difference spectra they produce when they combine with cytochrome P-450. In certain cases, the kinetics of the reactions between drugs and hemoprotein to produce spectral changes was sufficiently similar to the kinetics of the reactions involving the metabolism of the same drugs to suggest a causal relationship between binding to hemoprotein and drug metabolism. It was therefore of interest to study the effect of phospholipase C on the binding of drugs to cytochrome P-450 as well as on drug metabolism. Ethylmorphine, hexobarbital, and aniline were selected for study because much is known about their metabolism and because they react with microsomal hemoprotein to give representative types of binding spectra.

[2] The type I difference spectrum is characterized by an absorption peak at 385–390 mμ and a trough at 419–425 mμ; the type II difference spectrum has an absorption peak at 426–435 mμ and a trough at 390–405 mμ.

METHODS

Male Holtzman rats (250–280 g) were employed in all experiments. Hepatic microsomes were prepared as described previously (15) and were used on the day of their preparation.

Protein was determined by the method of Lowry *et al.* (16). Difference spectra produced by binding of ethylmorphine, hexobarbital, and aniline to cytochrome P-450 were determined as described by Remmer and associates (13), using a Shimadzu model MPS 50 dual-beam spectrophotometer. Cytochrome P-450 was determined essentially as described by Omura and Sato (3), using a Beckman model DB dual-beam spectrophotometer as described previously (15).

The composition of the incubation mixture for the measurement of the oxidative *N*-demethylation of ethylmorphine was the same as that described previously (17), except that nicotinamide was omitted. Microsomes (100,000 × *g* fraction) with a protein content of 4–5 mg/ml were incubated for 15 min at 37° with ethylmorphine (2 mM) in a volume of 5 ml. Aniline hydroxylase activity was determined as described by Kato and Gillette (18), with slight modifications of the incubation medium. The microsome fraction (100,000 × *g* pellet), with a protein content of 8–10 mg/ml, was incubated for 15 min at 37° with aniline (1 mM) in a volume of 5 ml. Microsomal hexobarbital oxidase activity was determined as described by Rubin *et al.* (19), except that a different liver preparation was used. Eight to ten milligrams of microsomal protein (100,000 × *g* pellet), plus 1.0 ml of 100,000 × *g* supernatant fraction, were incubated for 15 min at 37° with hexobarbital (0.6 mM) in a volume of 5 ml. The 100,000 × *g* supernatant fraction was added because maximum activity was not obtained with microsomes alone.

Phospholipase C (Sigma Chemical Company, type I, from *Clostridium welchii*, lot 38B-0150) incubations were carried out at room temperature (20–22°) in a medium containing 0.02 M Tris buffer (pH 7.4) and 2.5 mM $CaCl_2$. The protein concentration of

the microsomes was 4.2–5.0 mg/ml; the concentration of phospholipase C was varied according to the experiment. The phospholipase C reaction was terminated by the addition of EDTA (final concentration, 9 mM), which chelates the Ca^{++} needed for phospholipase C activity (20). After sedimenting the reaction mixture at 100,000 × *g* for 30 min, the pellet was washed once with 1.15% KCl solution containing 10 mM $MgCl_2$ and the suspension was again centrifuged at 100,000 × *g* for 30 min. The final pellet was suspended in sufficient KCl solution to give a protein concentration of 4–5 mg/ml.

Microsomal lipids were extracted by the method of Bligh and Dyer (21). The concentration of phospholipids was determined by the method of Bartlett (22). Phospholipids were separated by thin-layer chromatography on silica gel G, using chloroform–methanol–glacial acetic acid–water (65:25:8:4 by volume) as the developing system (23). The lipid spots were visualized by exposing the dried plates to iodine vapor for 2 min (24). The spots were circled and the iodine was allowed to evaporate. Quantitative determinations of the phospholipids were obtained by scraping the spots from the plates, transferring them to graduated conical centrifuge tubes, and eluting the lipids with warm chloroform–methanol solution (25). The solvent was removed with the aid of a stream of air, and the residues were assayed for total phosphorus (22).

The presence of phosphorylethanolamine and phosphorylcholine after treatment of microsomes with phospholipase C was determined by centrifuging the reaction mixture at 100,000 × *g* for 30 min, followed by mixing a suitable aliquot of the resulting supernatant fraction with an equal volume of cold 20% trichloracetic acid solution to destroy phospholipase C activity. After standing in the cold for 30 min, the mixture was centrifuged and the supernatant fraction was removed and evaporated under reduced pressure to a suitable volume. Aliquots were taken for phosphorus assay (22). An increase was seen in acid-soluble phosphorus in the phospholipase C-treated samples over that of control samples. This was due largely to the accumulation of phosphorylcholine and phosphorylethanolamine, as shown by paper chromatography using the developing solvent of Runeckles and Krotkov (26), which consists of ethylene glycol monomethyl ether–pyridine–glacial acetic acid–water (8:4:1:1 by volume).

RESULTS

Phospholipase C released 70% of the total phospholipids from microsomes; 79% of the phosphatidylcholine and 53% of the phosphatidylethanolamine in the microsomes were hydrolyzed (Table 1). As phosphatidylcholine and phosphatidylethanolamine disappeared from the microsomes, diglycerides accumulated in the microsomes, as shown by

TABLE 1

Effect of phospholipase C on microsomal phospholipids

Hepatic microsomes (4.2–5.0 mg of protein per milliliter) were digested with phospholipase C (0.8 mg/ml of incubation medium) in the presence of 0.02 M Tris buffer, pH 7.4, and 2.5 mM $CaCl_2$ at room temperature (20–22°) for 30 min. Figures in parentheses represent the number of experiments.

Assay	Phosphorus content		Loss
	Untreated microsomes (incubated in Tris buffer)	Phospholipase C-treated microsomes	
	μmole/mg protein ± SE		%
Total phospholipid phosphorus in 100,000 × *g* pellet (9)	0.428 ± 0.007	0.129 ± 0.007	70
Phosphatidylcholine in 100,000 × *g* pellet (9)	0.160 ± 0.005	0.034 ± 0.002	79
Phosphatidylethanolamine in 100,000 × *g* pellet (9)	0.062 ± 0.002	0.029 ± 0.002	53
Acid-soluble phosphorus in 100,000 × *g* supernatant (8)	0.066 ± 0.008	0.292 ± 0.018	

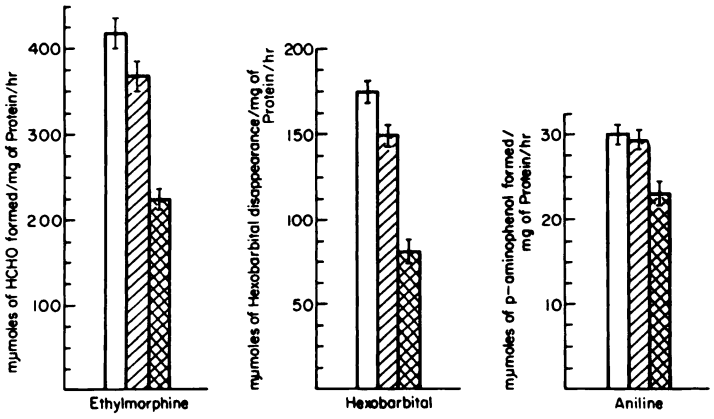

FIG. 1. *Effect of phospholipase C on microsomal drug metabolism*

Conditions for obtaining phospholipase C-treated microsomes are described in Table 1. The metabolism of ethylmorphine, hexobarbital, and aniline was determined as described in METHODS. Plain bars, microsomes not incubated but washed with KCl; singly hatched bars, microsomes incubated for 30 min at room temperature in 0.02 M Tris buffer and 2.5 mM $CaCl_2$; cross-hatched bars, phospholipase C-treated microsomes. Eleven ethylmorphine and aniline studies and three hexobarbital studies were performed; each study employed pooled livers from three rats. Vertical bars represent standard errors of the means.

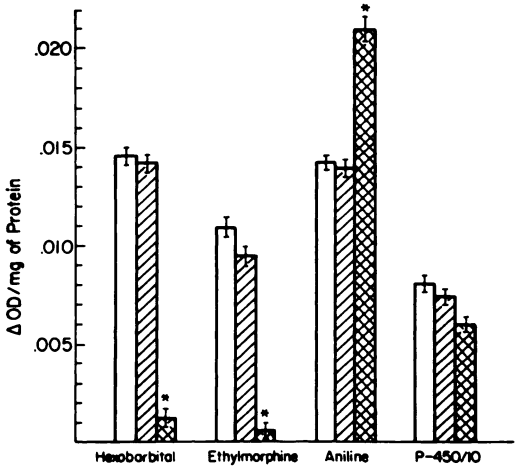

FIG. 2. *Effect of phospholipase C on binding of drugs to microsomal hemoprotein*

Conditions for obtaining phospholipase C-treated microsomes are described in Table 1. Difference spectra were obtained as described by Remmer *et al.* (13). Microsomes were diluted with 0.20 M Tris buffer (pH 7.4) to a protein concentration of 1 mg/ml. The final concentrations of drugs were: hexobarbital, 2.5 mM; ethylmorphine, 2.1 mM; and aniline, 6.7 mM. Difference spectra were obtained for hexobarbital and ethylmorphine between 420 and 500 mμ, for aniline between 430 and 500 mμ, and for P-450 between 450 and 500 mμ. Symbols represent the same conditions as in Fig. 1. Values are the means ± standard errors of 10 experiments, each of which employed the pooled livers from three rats.

* Significantly different from control ($p < 0.05$).

thin-layer chromatography on silica gel G using the developing solvent of petroleum ether–diethyl ether–glacial acetic acid (9:10:1 by volume) described by Malins and Mangold (24). The phosphorus that accumulated in the supernatant fraction as a result of treatment by microsomes with phospholipase (Table 1) was shown by paper chromatography (25) to be due largely to phosphorylcholine and phosphorylethanolamine.

Digestion of microsomes with phospholipase C caused a reduction of ethylmorphine and hexobarbital metabolism of 42 and 43%, respectively, but the metabolism of aniline was reduced by only 15% (Fig. 1). Hexobarbital and ethylmorphine show typical type I binding spectra when added to microsomes; aniline gives a typical type II spectrum. Figure 2 shows that the binding of both ethylmorphine and hexobarbital was virtually eliminated when microsomes were digested with phospholipase C, but the binding of aniline was increased. Phospholipase C caused a 20% loss of cytochrome P-450 (Fig. 2 and Table 3). In three determinations the change in absorbance between 450 and 500 mμ per milligram of protein of micro-

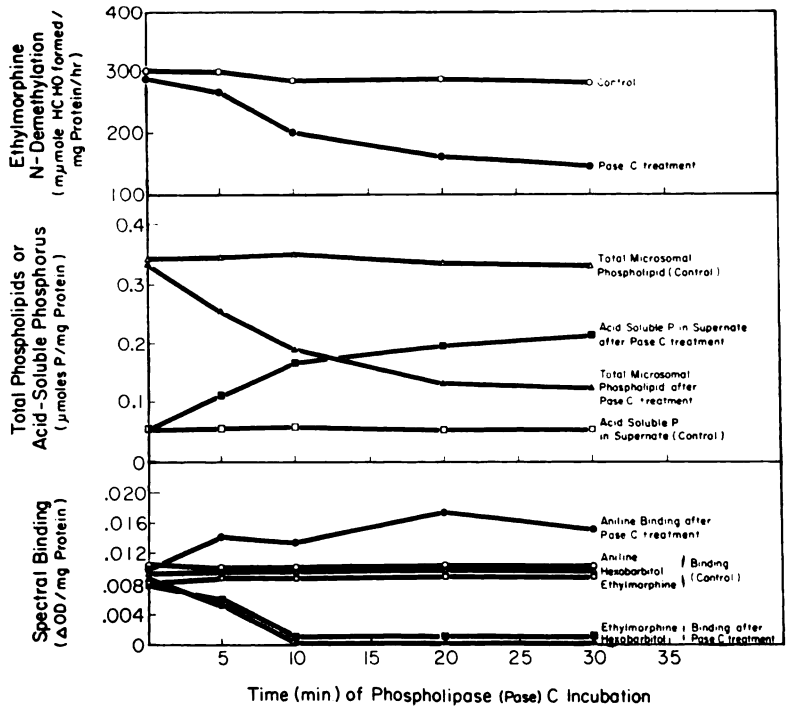

FIG. 3. *Effect of duration of incubation of microsomes with phospholipase C on microsomal N-demethylation of ethylmorphine, phospholipid content of microsomes, and binding of drugs to microsomal hemoprotein*

Microsomes (4.2–5.0 mg of protein per milliliter) were digested with phospholipase C (Pase C) (0.2 mg/ml of incubation medium) in the presence of 0.02 M Tris buffer (pH 7.4) and 2.5 mM $CaCl_2$ solution.

somes incubated with and without phospholipase C was 0.060 ± 0.002 and 0.076 ± 0.002, respectively, which represents a loss of about 20% of cytochrome P-450. The magnitude of the peak at 420 mμ seen in the spectrum obtained with phospholipase C-treated microsomes was compatible with a 20% conversion of cytochrome P-450 to cytochrome P-420. No cytochrome P-420 was detected in the medium from which these microsomes had been harvested.

To reinforce the impression given by the data presented in Table 1 and Figs. 1 and 2 that the losses of metabolism and binding of ethylmorphine and hexobarbital observed after microsomes had been incubated with phospholipase C were related to losses of phospholipids, microsomes were digested with phospholipase C for various times between 0 and 30 min. Figure 3 shows that the loss of total microsomal phospholipids with time correlates well with the losses of ethylmorphine metabolism and binding. The losses of hexobarbital and ethylmorphine binding were similar; aniline binding was again seen to increase after phospholipase C digestion. It is apparent that type I binding is not obligatory for the metabolism of ethylmorphine or hexobarbital, and that more than one pathway may therefore exist for the metabolism of these drugs. Further evidence that ethylmorphine may be metabolized by an alternative mechanism was obtained by determining the kinetic constants of ethylmorphine *N*-demethylation using phospholipase C-treated microsomes. A significant increase in apparent K_m and a decrease in apparent V_{max} were seen after microsomes were incubated with phospholipase C (Table 2).

To rule out the possibility that the effects of the phospholipase C preparation on the ability of microsomes to metabolize and bind drugs might be due to a contaminant in the preparation rather than to phospholipase C itself, Ca^{++} was omitted from the incubation

TABLE 2

Effect of phospholipase C treatment of microsomes on kinetic constants of ethylmorphine N-demethylation

Microsomes were digested with phospholipase C as described in Table 1. The kinetic constants were determined with the aid of statistical analysis (17). Numbers in parentheses indicate the number of experiments, each of which employed the pooled livers from three rats.

Treatment of microsomes	K_m	V_{max}[a]
	$M \times 10^4 \pm SE$	
None (incubated in Tris buffer)	2.86 ± 0.14 (9)	509.7 ± 48.1 (9)
Phospholipase C	6.93[b] ± 0.38 (11)	335.4[b] ± 24.7 (11)

[a] Nanomoles of formaldehyde formed per milligram of protein per hour ± standard error.
[b] Significantly different ($p < 0.05$) from results obtained with untreated microsomes.

TABLE 3

Effect of phospholipase C on microsomes in the presence and absence of Ca^{++}

Microsomes were digested with phospholipase C as described in Table 1, except that Ca^{++} was omitted where indicated. Control microsomes were incubated with Tris buffer in the absence of phospholipase C. Values are the means ± standard errors of three experiments, each of which employed the pooled livers of three rats.

Assay	Control (with Ca^{++})	Phospholipase C-treated (with Ca^{++})	Control (no Ca^{++})	Phospholipase C-treated (no Ca^{++})
Ethylmorphine metabolism[a]	357.5 ± 5.2	204.7 ± 16.7 (−43%)	372.9 ± 27.2	369.4 ± 15.9
Hexobarbital metabolism[b]	112.7 ± 14.0	41.7 ± 9.3 (−63%)	133.4 ± 10.0	140.4 ± 13.3
Aniline metabolism[c]	28.0 ± 1.6	19.2 ± 2.4 (−31%)	28.0 ± 2.9	28.7 ± 1.6
Ethylmorphine binding[d]	0.0073 ± 0.0003	0	0.0087 ± 0.0017	0.0088 ± 0.0012
Hexobarbital binding[d]	0.0073 ± 0.0003	0	0.0093 ± 0.0009	0.0081 ± 0.0004
Aniline binding[e]	0.0134 ± 0.0009	0.0210 ± 0.0026	0.0134 ± 0.0024	0.0137 ± 0.0023
P-450[f]	0.076 ± 0.002	0.056 ± 0.008	0.078 ± 0.011	0.078 ± 0.005
Total phosphorus in 100,000 × *g* pellet[g]	0.42 ± 0.05	0.14 ± 0.02 (−67%)	0.39 ± 0.03	0.45 ± 0.05
Acid-soluble phosphorus in 100,000 × *g* supernatant[g]	0.086 ± 0.004	0.309 ± 0.017	0.073 ± 0.002	0.085 ± 0.004

[a] Nanomoles of formaldehyde formed per milligram of protein per hour.
[b] Nanomoles of hexobarbital disappearance per milligram of protein per hour.
[c] Nanomoles of *p*-aminophenol formed per milligram of protein per hour.
[d] Change in absorbance between 420 and 500 mμ per milligram of protein.
[e] Change in absorbance between 430 and 500 mμ per milligram of protein.
[f] Change in absorbance between 450 and 500 mμ per milligram of protein.
[g] Micromoles of phosphorus per milligram of microsomal protein.

medium. Ca^{++} is required for phospholipase C activity (20). Without Ca^{++}, phospholipase C had no effect on the metabolism or binding of ethylmorphine, hexobarbital, or aniline, the loss of P-450, or the phospholipid content of the microsomes (Table 3).

It was also important to rule out the possibility that the products of the phospholipase C reaction (water-soluble phosphoryl compounds and diglycerides) inhibit the metabolism of ethylmorphine and hexobarbital, interfere with type I binding, or bind as type I compounds so as to obscure type I binding of ethylmorphine or hexobarbital. Phosphorylcholine and phosphorylethanolamine are water-soluble and remain in the

100,000 × *g* supernatant fraction of phospholipase C-digested microsomes. Washing the phospholipase C-digested microsomal pellet repeatedly with 1.15% KCl solution did not restore the activity lost as a result of phospholipase C treatment. Phosphorylcholine and phosphorylethanolamine added to untreated microsomes in the high concentrations of 2.0 and 2.8 mM, respectively, had no effect on the metabolism of ethylmorphine, hexobarbital, or aniline. They did not interfere with the binding spectra of hexobarbital, ethylmorphine, or aniline, nor did they produce type I or type II binding spectra when added to microsomes. No attempt was made to remove the diglycerides from phospholipase C-treated microsomes; however, the addition of diglyceride (10 μmoles) to the incubation mixture containing untreated microsomes had no effect on the metabolism of ethylmorphine.

DISCUSSION

The importance of the association of microsomal hemoprotein with membrane lipid became known largely as a result of studies in which cytochrome P-450 was converted to cytochrome P-420, a spectrally altered, inactive form of the hemoprotein. Cytochrome P-450 is converted to cytochrome P-420 by a wide variety of agents: snake venom containing phospholipase A (1–3); detergents such as sodium deoxycholate (2, 3) and lysolecithin (4); the metal-binding reagents bathocuproinesulfonate and bathophenanthrolinesulfonate (27, 28); trypsin (27, 29); urea (27) and urea derivatives (30); guanidine (4); neutral salts (4); the mercurial sulfhydryl-binding agents *p*-chloromercuribenzoate (27), *p*-chloromercuriphenylsulfate (27), and $HgCl_2$ (27, 31); iodine (4, 32); aniline (4); alcohols (4, 27, 30); and a variety of amides and phenols (30). In a critical analysis of the manner in which these diverse agents convert cytochrome P-450 to cytochrome P-420, Imai and Sato (4) concluded that the ultimate effect of each agent was to disrupt the association between hemoprotein and microsomal lipid. Depending upon the agent, this might occur either by its action on the lipid or by an effect on protein associated with the lipid. Since with few exceptions it is those drugs possessing high lipid solubility which react with the drug-metabolizing system, the active area of cytochrome P-450 was assumed by Imai and Sato to be in contact with or "embedded" in a highly hydrophobic part of cytochrome P-450 protein or in lipids of the microsomal membrane.

Remmer and co-workers (13) and Imai and Sato (14) showed that two types of spectral change (I and II) result when various drugs combine with microsomal hemoprotein. The magnitudes of these spectral changes were found to depend upon the concentrations of substrate and microsomal protein. Schenkman *et al.* (33) proposed that the binding of substrates to microsomal hemoprotein precedes enzymatic conversion of the substrate, and therefore the appearance of a spectral change might be regarded as evidence for the formation of an enzyme-substrate complex. This postulate was based largely on the observation that the spectral dissociation constants and the Michaelis constants of two of the substrates examined (hexobarbital and aminopyrine) were nearly the same. It was further proposed that substrates which produce the type II spectral change interact with the iron at the CO-binding site of the heme, because aniline, a type II compound, displaced CO from the CO–cytochrome P-450 complex. The interaction of type I compounds was considered to take place at a different site, since addition of hexobarbital did not displace CO. Schenkman and Sato (34) postulated that the type I binding spectrum may result when type I compounds displace the sixth ligand of the heme from a hydrophobic region of the apoenzyme. Our observation that digestion of microsomes with phospholipase C destroys type I binding, but does not decrease type II binding, provides further evidence that the two binding sites differ and supports the view that the type I binding site is closely associated with membrane phospholipids. It is probable that phospholipids are essential for the specific conformation of the type I binding site. They may even be an integral part of the sixth ligand to the heme of cytochrome P-450. The failure of phospholipase C to destroy type II binding indicates either that phospholipids are not needed to preserve the integrity of the type II site or that the site is

associated with phospholipid in such a way that it is impervious to the action of phospholipase C.

The observation that phospholipase C-treated microsomes can metabolize ethylmorphine and hexobarbital without exhibiting a type I binding spectrum is not consistent with the view that binding is an obligatory step in the mechanism of drug hydroxylation and suggests the involvement of an alternative metabolic route. However, the current studies do not rule out a role of type I binding in the oxidation of type I drugs. If the reactivity of type I compounds with an alternative system is less than that with the system involving type I binding, as may be indicated by the increase in the apparent K_m of ethylmorphine *N*-demethylation observed after micorsomes are treated with phospholipase C (Table 2), the alternative route of metabolism may represent a minor pathway when the type I binding site is intact and thus might not greatly influence the kinetics of the over-all reaction when untreated microsomes are employed. On the other hand, metabolism of type I compounds by two routes, one of which involves the type I binding site and one of which does not, need not necessarily involve different enzyme systems. As judged from the inhibitory effect of carbon monoxide, both routes require cytochrome P-450. Carbon monoxide (atmosphere, 80% CO–20% air) inhibited the *N*-demethylation of ethylmorphine by untreated and phospholipase C-treated microsomes 87 and 92%, respectively (atmosphere used in control incubations, 80% N_2–20% air). If a single enzyme system is involved in the oxidation of type I compounds, the role of the type I binding site might be thought of as facilitative rather than obligatory. A facilitative role of the type I site is not difficult to visualize. According to Imai and Sato (4), binding spectra result when drugs combine with hemoprotein to produce conformational changes in the protein. These conformational changes might well facilitate the flow of electrons within the drug-hemoprotein complex so as to enhance the reaction rate. Evidence for such a facilitative role of the type I binding site was provided by studies of Gillette and co-workers (35–38) and by Schenkman (39), who observed that type I compounds stimulated NADPH–cytochrome P-450 reductase activity. When the type I site is not available, as is the case after microsomes have been treated with phospholipase C, the drug-induced conformational change in the hemoprotein is not possible. Nevertheless, the interaction between drug and hemoprotein occurs; hydroxylation takes place, but less readily than when the type I site is functional. As Imai and Sato (40) have pointed out, benzene is metabolized by the microsomal system even though it does not produce a binding spectrum with microsomes. A second possibility that could explain metabolism of type I compounds in the absence of type I binding is that two cytochrome P-450s may exist, only one of which is associated with phospholipid to form a type I binding site. Both cytochromes would participate in the hydroxylation reaction, but the cytochrome P-450 with the type I binding site would be the more reactive of the two. A third, but least likely, possibility that must be considered is that type I binding may have little if anything to do with drug metabolism.

Using aniline as the type II compound, the magnitude of the type II binding spectrum was observed to be augmented after microsomes had been incubated with phospholipase C. This can be explained if aniline combines not only with the type II binding site, but also, to a lesser degree, with the type I binding site. Type I and type II binding spectra are near mirror images. Thus, when difference spectra are being observed, the maximum and minimum peaks of each spectrum tend to oppose each other, and it is thus not possible to determine how much affinity a type II compound has for the type I binding site and vice versa. If a compound combined with both binding sites, the type I and type II spectra would tend to cancel each other; only that spectrum would be seen which represented the binding site most able to combine with the compound. In accordance with this concept, phospholipase C removes the type I binding site, and the type I binding with aniline that would normally have occurred in untreated microsomes is no longer present to produce a spectrum that subtracts from the type II spectrum formed with aniline; hence, the type II spectrum is

augmented after phospholipase C incubation. Evidence has been presented by several investigators suggesting that many compounds may combine in varying degrees with both binding sites (41–43). Alternative to this concept, the ligand state of the hemoprotein may be affected by its association in the membrane with phospholipid. When it is released from this association, new, more favorable association constants for type II compounds are created.

The observation that treatment of microsomes with phospholipase C destroys all type I binding with only a 20% conversion of cytochrome P-450 to cytochrome P-420 could mean that only 20% of the total microsomal cytochrome P-450 is associated with membrane phospholipid in a manner that enables the formation of a type I binding site. This would support the contention of Ullrich (32) that only a small percentage of the cytochrome P-450 in microsomes is available for type I drug binding. This could mean that only a small part of cytochrome P-450 is associated with phospholipid, or that two cytochrome P-450s exist, only one of which is bound to phospholipid. It is also conceivable that cytochrome P-450 might lose type I binding sites as a result of phospholipase C treatment without being converted to cytochrome P-420.

To establish unequivocably the essentiality of lipid in the microsomal drug-metabolizing complex, it is necessary not only to correlate loss of enzymatic activity with alteration or removal of lipid, but also to demonstrate restoration of activity after addition of lipid to the treated microsomes (44). Although our data show that partial loss of enzymatic activity and loss of binding can be correlated with a loss of microsomal phospholipids, attempts to re-establish lost activities and binding were not successful. It is possible that conformational changes in cytochrome P-450 occur when the hemoprotein is dissociated from phospholipid which cannot be reversed readily.

Of considerable interest with respect to the current studies is the recent observation made in Coon's laboratory of a phosphatidylcholine requirement in the hydroxylation of drugs by a solubilized microsomal system containing cytochrome P-450 (45). Previous studies from that laboratory showed that hepatic microsomes from the rabbit which had been solubilized using a mixture of glycerol, dithiothreitol, and sodium deoxycholate could be resolved into a fraction containing cytochrome P-450, a fraction containing an NADPH reductase, and a heat-stable, lipid fraction (46–48). All three fractions were necessary for the maximal oxidation of drugs. Phosphatidylcholine has now been shown to substitute for the lipid fraction. Benzphetamine and ethylmorphine, the drug substrates used in these studies, are known to be compounds which give a type I binding spectrum with microsomes.

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