A Role for Phospholipids in the Binding and Metabolism of Drugs by Hepatic Microsomes

USE OF THE FLUORESCENT HYDROPHOBIC PROBE 1-ANILINONAPHTHALENE-8-SULPHONATE

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(Received 19 March 1971)

1. The pretreatment of rat liver microsomes with phospholipase C or D decreased the *N*-demethylation of (+)-benzphetamine. The hydroxylation of aniline was essentially unchanged by pretreatment of microsomes with phospholipase C. 2. Some components of the microsomal mixed-function oxidase system were impaired by phospholipases. 3. The fluorescence of 1-anilidonaphthalene-8-sulphonate (ANS) was greatly enhanced by microsomes. Phospholipase C or D markedly decreased ANS-microsome fluorescence. Quantum yield of ANS-microsome fluorescence appeared to be related directly to phospholipid content of microsomes. 4. Most of the drugs studied enhanced ANS-microsome fluorescence. Warfarin, however, displaced ANS fluorescence competitively from microsomes. The latter effect was postulated as being due to warfarin competing with ANS for the cationic site on microsomal phosphatidylcholine. 5. ANS fluorescence was also increased by the presence of phospholipid micelles. The fluorescence of ANS-phosphatidylcholine micelles was modified by warfarin and (+)-benzphetamine in a manner similar to that observed with microsomes. Warfarin decrease of fluorescence was absent when ANS was bound to phosphatidic acid, which lacks a cationic site. 6. Trypsin pretreatment of microsomes did not modify ANS-microsome fluorescence, including drug-induced changes. 7. It was postulated that phospholipids have a permissive role in the metabolism of most drugs by hepatic microsomes and that the ANS probe might reflect interactions of compounds with microsomal membrane phospholipids.

It is well known that a large number of foreign and endogenous compounds are metabolized in mammals by a mixed-function oxidase system localized in the microsomal membranes of the hepatocyte (Gillette, 1966). This enzyme system is considered to utilize a flavoenzyme, NADPH-cytochrome *c* reductase, and the haemoprotein, cytochrome *P*-450. Estabrook & Cohen (1969) have suggested also a role for cytochrome *b*₅ in this electron-transport scheme.

Another characteristic of hepatic microsomal membranes is that they contain relatively large amounts of phospholipids, especially phosphatidylcholine, with smaller amounts of triglycerides,

cholesterol, cholesteryl esters and free fatty acids (Glaumann & Dallner, 1968). Moreover, enzymes for the synthesis (Kornberg & Pricer, 1953; Stein & Shapiro, 1957; Bremer & Greenberg, 1961; Gibson, Wilson & Udenfriend, 1961; Wilgram & Kennedy, 1963; Lands & Hart, 1964; Daae & Bremer, 1970) and degradation (Bjørnstad, 1966; Waite & Van Deenen, 1967) of phospholipids are present in microsomes.

A number of workers have demonstrated a phospholipid-dependency for microsomal enzymes such as adenosine triphosphatase (Martonosi, Donley & Halpin, 1968), NADH-cytochrome *c* reductase (Dallner, Siekevitz & Palade, 1965; Jones & Wakil, 1967), glucose 6-phosphatase (Duttera, Byrne & Ganoza, 1968) and UDP-glucuronyl-transferase (Graham & Wood, 1969). A suggested catalytic function for membrane phospholipids is that they serve as bridges between aqueous, or hydrophilic, and non-aqueous, or hydrophobic,

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functional groups, in addition to any role they have as molecular 'cement' or determinants of the tridimensional structure of membrane proteins (cf. Triggle, 1969).

By using the hydrophobic fluorescent probe ANS† in addition to lipolytic and proteolytic enzyme treatments, a study was undertaken to investigate a possible role for phospholipids in the binding and metabolism of drugs by hepatic microsomal membranes. Previously, phospholipids have been implicated in ANS-membrane interactions by using muscle (Vanderkooi & Martonosi, 1969) and brain (Mayer & Avi-Dor, 1969) microsomal preparations, mitochondrial membrane fragments (Azzi, Chance, Radda & Lee, 1969) and erythrocyte membranes (Rubalcava, DeMunoz & Gitler, 1969).

MATERIALS AND METHODS

Microsomes were prepared from male Sprague-Dawley rats (150–200 g) fed *ad libitum*. The rats were killed by cervical dislocation, and the livers removed and immersed in 0.15 M-KCl, pH 7.0–7.4. All subsequent operations were made at 0–4°C. The tissue was minced, washed repeatedly in KCl solution and then homogenized in 2 vol. of the KCl solution with a Potter-Elvehjem-type homogenizer equipped with a Teflon pestle.

The homogenates were centrifuged for 20 min at 9000g in a Sorvall RC2-B refrigerated centrifuge. The 9000g supernatant was carefully withdrawn with a syringe. Microsomes were isolated by centrifugation of the 9000g supernatant fraction at 60000 rev./min for 30 min in an IEC B-60 refrigerated centrifuge with an A-321 rotor. The microsomal pellets were resuspended in the KCl solution and resedimented. Microsomal protein content was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as the standard.

For the treatment of hepatic microsomes with trypsin, the microsomes, suspended in 0.15 M-KCl, were diluted to a final protein and potassium phosphate buffer concentration of 7.5 mg/ml and 0.1 M respectively. Bovine pancreatic trypsin (Sigma Chemical Co., St Louis, Mo., U.S.A.) was added to give a final concentration of 50 µg of enzyme/mg of microsomal protein. The final mixture was incubated at 37°C for 30 min under an atmosphere of N₂ in a Dubnoff metabolic shaker. After the digestion period, the mixtures were chilled on ice and centrifuged at 60000 rev./min as stated above. The pellets obtained were stored at 4°C overnight under N₂.

For the treatment of microsomes with phospholipase C the microsomes were diluted to 5 mg of protein/ml in 0.1 M-HEPES (*N*-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) buffer, pH 7.4, containing 1 mM-CaCl₂. Phospholipase C (type I; Sigma Chemical Co.) from *Clostridium welchii* was added as a suspension in the CaCl₂-HEPES buffer to give a final concentration of 0.15 mg of the enzyme/mg of microsomal protein. Digestion was made at 0°C for 30 min. Digested microsomes were sedimented at 60000 rev./min as described above.

† Abbreviation: ANS, 1-anilinonaphthalene-8-sulphonate.

In some experiments the pellets were stored at 0°C under N₂ for use the following day. The phospholipid phosphorus content of microsomes was measured by using a combination of the methods of Shin (1962) and Fiske & Subbarow (1925). The amount of phospholipid was determined by multiplying the amount of phospholipid phosphorus by 25.

The incubation media for phospholipase D digestion contained microsomes at about 7 mg of protein/ml in 30 mM-acetate buffer, pH 5.7, containing 0.4 M-CaCl₂. Cabbage phospholipase D (type I; Sigma Chemical Co.) was dissolved in the acetate buffer to give a final concentration of 0.1 mg of the enzyme/mg of microsomal protein. The mixture was incubated at 37°C for 30 min in a Dubnoff shaker. After incubation, duplicate 1 ml samples were added to 1 ml of 9% (w/v) HClO₄ for the determination of liberated choline by the method of Appleton, La Du, Levy, Steele & Brodie (1953), as modified by Dawson (1956). The remaining suspension was sedimented at 60000 rev./min as described above.

By using a method similar to that of Anson & Mirsky (1933) with bovine albumin as a substrate, no detectable proteolytic activity was found in the preparations of phospholipase C and phospholipase D purchased. Accordingly, trypsin produced a considerable amount of 'Folin-Ciocalteu-positive' material.

The incubation mixtures for drug metabolism experiments *in vitro* consisted of microsomes (2.0 mg of protein), 0.25 ml of the supernatant (post-microsomal) fraction obtained from untreated microsomes, 5 mM-MgSO₄, 5 mM-glucose 6-phosphate, 0.4 mM-NADP⁺ [for (+)-benzphetamine or (+)-*N*-benzyl-*N*, α -dimethylphenethylamine hydrochloride metabolism] or 1.0 mM-NADP⁺ [for aniline hydrochloride metabolism], 5 mM-semicarbazide (for formaldehyde assay), substrate [10 mM-(+)-benzphetamine or 2.0 mM-aniline] and 50 mM-phosphate buffer, pH 7.4, in a total volume of 2.5 ml. The conditions of incubation and methods of analyses have been described by DiAugustine & Fouts (1969).

NADPH-cytochrome *c* reductase was measured as described by Phillips & Langdon (1962). NADPH-cytochrome *P*-450 reductase was measured by the method of Gigon, Gram & Gillette (1969); however, the reaction mixture was maintained at 26°C rather than 37°C.

Extinction measurements were made with a Shimadzu MPS-50L split-beam recording spectrophotometer; samples were kept at room temperature during the experiments. Cytochrome *P*-450 and cytochrome *b*₅ were determined by the method of Omura & Sato (1964). The type I and type II difference spectra were obtained by the addition of (+)-benzphetamine or aniline to microsomal suspensions. Extinction was measured as the difference between the trough at 420 nm and the peak at 385–390 nm for (+)-benzphetamine (type I), or the trough at 395 nm and the peak at 430 nm for aniline (type II).

Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorimeter (model 4-8202) equipped with a 150 W Hanovia Xenon arc lamp. Measurements were carried out at 25°C with a 0.1 ml quartz cell (3 mm × 3 mm internal × 20 mm high). The excitation wavelength was 378 nm; ANS (ammonium salt) was purchased from Pierce Chemical Co., Rockford, Ill., U.S.A., and was used without further purification. In most fluorescence experiments, the microsomal suspension

at a concentration of 4 mg of protein/ml was mixed with an equal volume of the drug in 0.1M-phosphate buffer, pH 7.4. A sample of this solution was then added to the cell with a Pasteur pipette and fluorescence emission was measured. The concentrations of ANS used in aqueous solutions were kept at or below 100 μ M, since above this concentration an inner filter effect was observed (Di-Augustine, Eling & Fouts, 1970). In studies where butan-1-ol was used as a solvent, the fluorescence of ANS was proportional to concentration up to approx. 50 μ M.

Phospholipids were dispersed ultrasonically by using a Sonifier Cell Disrupter (Branson Instruments Inc., Melville, N.Y., U.S.A.). The phospholipids (1 mg/ml) were placed in a 50 ml beaker with an appropriate amount of 50 mM-phosphate buffer, pH 7.4, and sonicated over cracked ice at approx. 100 W for 4 min. The phospholipid micelles were used for about 8 h without repeated ultrasonic treatment. Bovine phosphatidylcholine, (distearoyl) L- α -phosphatidylcholine and (dioleoyl) L- α -phosphatidylcholine were purchased from Supelco Inc., Bellefonte, Pa., U.S.A., phosphatidic acid (sodium salt) from egg phosphatidylcholine was purchased from General Biochemicals, Chagrin Falls, Ohio, U.S.A.

For the determination of ANS-microsome fluorescence in the presence of phospholipase C the medium contained microsomes (1.0 mg of protein/ml), 100 μ M-ANS, 1.0 mM-

CaCl₂ and phospholipase C (0.15 mg of enzyme/mg of microsomal protein) in 0.1M-HEPES buffer, pH 7.4. After the addition of the phospholipase the mixture was rapidly transferred to the quartz cell and fluorescence emission intensity was measured at 475 nm. Simultaneous determinations of residual phospholipid phosphorus were made in the manner described above.

RESULTS

Effects of phospholipases on microsomal drug metabolism. The incubation of rat hepatic microsomes with phospholipase C (EC 3.1.4.3) modified components of the microsomal electron-transport system and also changed drug-induced microsomal spectral changes. As summarized in Table 1, the metabolism of aniline, a type II ligand, NADPH-cytochrome *c* reductase and cytochrome *b₅* content were not changed by phospholipase C treatment. The small decrease in cytochrome *P*-450 was paralleled by the appearance of cytochrome *P*-420. The metabolism of (+)-benzphetamine, the (+)-benzphetamine-induced, or type I, spectral change and NADPH-cytochrome *P*-450 reductase were

Table 1. *Effects of phospholipases C and D on drug metabolism, cytochromes and spectral characteristics of rat hepatic microsomes*

	Phospholipase C		Phospholipase D	
	Control	Treated	Control	Treated
(+)-Benzphetamine <i>N</i> -demethylase (nmol of formaldehyde/30 min per mg of microsomal protein)	200	70	60	8
Aniline hydroxylase (nmol of <i>p</i> -aminophenol/30 min per mg of microsomal protein)	21	22	16	5
(+)-Benzphetamine (type I) maximum spectral change (ΔE /mg of microsomal protein)	0.016	0.005	0.016	0.008
Aniline (type II) maximum spectral change (ΔE /mg of microsomal protein)	0.025	0.050	0.023	0.032
Cytochrome <i>P</i> -450 (nmol/mg of microsomal protein)	1.07	1.00	0.75	0.70
Cytochrome <i>b₅</i> (nmol/mg of microsomal protein)	0.48	0.45	0.75	0
NADPH-cytochrome <i>P</i> -450 reductase (nmol/min per mg of microsomal protein)	1.7	0.5	1.3	0.2
NADPH-cytochrome <i>c</i> reductase (nmol/min per mg of microsomal protein)	24.3	21.0	10.0	1.6

decreased by 60–70%. The type II spectral change produced by aniline was increased in phospholipase C-treated microsomes.

Phospholipase D treatment only slightly decreased the cytochrome *P*-450 content of hepatic microsomes; the metabolism of (+)-benzphetamine and aniline, the (+)-benzphetamine-induced spectral change, NADPH-cytochrome *P*-450 reductase and NADPH-cytochrome *c* reductase were markedly decreased (Table 1). The aniline-induced spectral change was increased as a result of digestion with phospholipase D. Cytochrome *b*₅ was apparently completely absent from phospholipase D-treated microsomes. The latter effect is in disagreement with that of Lumper, Zabrzycki & Staudinger (1969), who reported that phospholipase D (EC 3.1.4.4) did not release cytochrome *b*₅ from microsomal membranes. However, these results might be explained by differences in the preparation of phospholipase D, since this enzyme, as purchased, is impure. A striking finding was the large increase in cytochrome *b*₅ in microsomes after incubation in acetate buffer at pH 5.6. Similarly, Hewick & Fouts (1970) found that the content of cytochrome *b*₅ (per mg of microsomal protein) apparently increased when microsomal preparations were stored in phosphate buffer, pH 7.4, at 1°C; during aging, cytochrome *P*-450, drug-induced spectral changes and drug metabolism by microsomal preparations decreased.

The acidic conditions used for phospholipase D digestion probably denatured some microsomal-membrane components, which would explain the differences obtained in phospholipase C and phospholipase D controls.

Effects of phospholipases on the interaction of ANS with microsomes. The fluorescent dye ANS interacted with hepatic microsomes to yield a large enhancement of fluorescence and a 45nm hypsochromic shift in the peak of the ANS emission spectrum. Digestion of microsomes with phospholipase C decreased ANS-microsome fluorescence by approx. 60% and caused a further hypsochromic shift (475 to 470nm) in the peak of the ANS-microsome emission spectrum. The phospholipid phosphorus content of microsomes also was decreased by approx. 60% as a result of phospholipase C treatment. Incubation of microsomal preparations with phospholipase C beyond 30min yielded no further loss of phospholipid phosphorus. These results were obtained when the digestion was made at 0 or 37°C. However, whereas no protein was lost at 0°C, digestion at 37°C gave about a 25% loss of microsomal protein. The latter effect was probably due to conditions favourable to the release of microsomal intraluminal protein (Glaumann & Dallner, 1968).

Digestion of microsomes with phospholipase D

Fig. 1. Double-reciprocal plots of ANS fluorescence against microsomal protein concentration. ● and ▲, 10 μ M-ANS; ○ and △, 20 μ M-ANS. —, Control microsomes; ----, phospholipase-treated microsomes. (a) Phospholipase C; (b) phospholipase D. Microsomes were adjusted to a protein concentration of 1 mg/ml.

decreased ANS-microsome fluorescence by approx. 30% and produced a 10nm hypsochromic shift (475 to 465nm). Treatment at 37°C with phospholipase D did not result in significant protein loss when compared with controls. The determination

Fig. 2. Scatchard plots for the fluorescence of ANS bound to rat hepatic microsomes. ▲, Control microsomes; ●, phospholipase-treated microsomes. (a) Phospholipase C; (b) phospholipase D. Microsomes were adjusted to a protein concentration of 1 mg/ml.

of choline as a soluble by-product of phospholipase D activity by the method of Appleton *et al.* (1953) revealed the presence of a considerable amount of non-choline material which was also assayed as choline. It is possible that this material represents other bases or acids liberated from microsomal phospholipids by phospholipase D (Heller & Arad, 1970). By using incubation conditions similar to those we employed, Lumper *et al.* (1969) reported that microsomes labelled *in vivo* with [^{14}C]choline lost 80% of their radioactivity upon treatment with phospholipase D.

Double-reciprocal plots of ANS fluorescence against microsomal protein concentration were

made for control, phospholipase C- and phospholipase D-treated microsomal preparations. The fluorescence at infinite protein concentration can be obtained from the extrapolated intercepts on the ordinate. As shown in Fig. 1, the intercepts were identical for control and treated microsomes. This indicated that the fluorescent enhancement factor for the ANS interaction with microsomes was not modified by enzyme treatment. The decrease in fluorescence obtained with phospholipases is more likely attributed to a smaller pool size of binding sites without changes in the enhancement factor for ANS bound to microsomes.

By using the enhancement factor of 250, the ANS-microsome interaction was further interpreted by a Scatchard (1949) plot as described by DiAugustine *et al.* (1970). The control plot of the ANS interaction with microsomes resulted in a curve which indicated that there is more than one class of ligand sites for ANS on microsomes (Fig. 2a). However, the Scatchard plot for phospholipase C-treated microsomes was significantly less curvilinear than that of untreated controls; the enzyme treatment greatly altered the ANS interaction with microsomes by removing nearly all the high-affinity sites without changing markedly the number of low-affinity sites. A Scatchard plot of the ANS fluorescent interaction with microsomes treated with phospholipase D revealed a smaller proportion of high-affinity sites (Fig. 2b); the number of low-affinity binding sites was not altered. The plots for phospholipase C- and phospholipase D-treated microsomes were similar in that both had more linear slopes by destruction of a high-affinity binding site.

A comparison of the Scatchard plots for control microsomes shows that the heating of microsomes at 37°C at a pH of 5.6 (conditions for optimum hydrolysis by phospholipase D) modified the interaction of ANS with microsomes. Also, incubated controls (37°C) generally gave a smaller relative fluorescence than non-incubated (0°C) controls. Scatchard plots revealed that the high-affinity sites were specifically depleted by incubation.

Effects of drugs on the fluorescence of ANS-microsome mixtures. As reported by DiAugustine *et al.* (1970), the fluorescence of ANS-microsome mixtures was modified by the presence of compounds used as substrates or inhibitors of hepatic microsomal drug metabolism. (+)-Benzphetamine, a substrate, and 2,4-dichloro-6-phenylphenoxyethylamine hydrobromide (DPEA, Lilly 53325), 2,4-dichloro-6-phenylphenoxyethyldiethylamine hydrobromide (Lilly 18947) and β -diethylaminoethyl diphenylpropylacetate hydrochloride (SKF-525A), inhibitors, all increased ANS-microsome fluorescence. Sodium warfarin, a substrate, was shown to

be a competitive inhibitor of ANS-microsome fluorescence at concentrations above 1 mM. Digestion of microsomes with phospholipase C or phospholipase D modified the effect of these compounds on ANS-microsome fluorescence. As shown in Fig. 3, (+)-benzphetamine enhanced the relative fluorescence of ANS for both phospholipase C- and phospholipase D-treated microsomes to a greater extent than observed with untreated microsomes, even though ANS-microsome fluorescence was less than that obtained with untreated controls. (+)-Benzphetamine increased ANS-microsome fluorescence in phospholipase C-treated microsomes nearly threefold. DPEA, Lilly 18947 and SKF-525A enhanced the ANS-microsome fluorescence with phospholipase-digested microsomes in the manner observed with (+)-benzphetamine.

From double-reciprocal and Scatchard plots, warfarin appeared to be a competitive inhibitor of ANS-microsome fluorescence, displacing the ANS probe from the high-affinity site, but leaving intact the ANS fluorescence due to interaction at the low-affinity site (DiAugustine *et al.* 1970). The enzymatic activities of phospholipases C and D appear to yield a warfarin-type action in that they obliterate high-affinity binding sites. Thus it

should seem reasonable that the phospholipases decrease the effectiveness of warfarin as an inhibitor of ANS-microsome fluorescence. At an ANS concentration of $3\mu\text{M}$, warfarin (10 mM) inhibited ANS fluorescence by approx. 40–50%; after phospholipase digestion, warfarin decreased ANS-microsome fluorescence by 20–25% (Fig. 3). The warfarin inhibition of fluorescence of ANS bound to microsomes treated with phospholipases is shown by the reciprocal plots in Fig. 4. The plots became curvilinear after phospholipase treatment, especially with type C.

None of the chemicals or enzymes at the concentrations reported gave significant changes of fluorescence of ANS in aqueous solutions. In accordance with the results of Stryer (1965) quantum yield of fluorescence of ANS in butan-1-ol greatly increased and was accompanied by a hypsochromic shift in the emission spectrum similar to that observed in the presence of microsomes. However, 1 mM-(+)-benzphetamine, as well as 10 mM-warfarin, inhibited ANS fluorescence by 15–20% in the non-polar environment offered by this alcohol. Unlike that observed with microsomes, the percentage inhibition of ANS-butan-1-ol fluorescence remained constant regardless of the concentration of ANS. Thus it is difficult to ascribe

Fig. 3. Fluorescence of ANS-microsomes in the presence of warfarin or (+)-benzphetamine. Δ and $\blacktriangle$, ANS; $\circ$ and $\bullet$, ANS plus 1 mM-(+)-benzphetamine; $\square$ and $\blacksquare$, ANS plus 10 mM-warfarin. ----, Control microsomes; —, phospholipase-treated microsomes; (a) phospholipase C; (b) phospholipase D. Microsomes were adjusted to a protein concentration of 1 mg/ml.

Fig. 4. Double-reciprocal plots of ANS-microsome fluorescence against ANS concentration. —, Control microsomes; ----, phospholipase-treated microsomes; (a) phospholipase C; (b) phospholipase D. ▲, ANS control; △, ANS plus 10 mM-warfarin; ●, ANS; ○, ANS plus warfarin after phospholipase treatment. Microsomes were adjusted to 1 mg of protein/ml.

changes of ANS-microsome fluorescence produced by (+)-benzphetamine or warfarin to a random interaction or simple partitioning of the probe and substrate in a hydrophobic phase of the microsomal membrane.

Simultaneous time-course determination of ANS-microsome fluorescence and phospholipid P release by phospholipase C action. The ratio of ANS-microsome fluorescence to microsomal phospholipid content did not appear to change after phospholipase C treatment of microsomes. To examine further the interdependency between ANS fluorescence and microsomal phospholipids, the decay of ANS-microsome fluorescence and the amount of residual phospholipid phosphorus were measured simultaneously. Fig. 5 shows that during the first 2 min of digestion of phospholipase C, the slopes of the lines for relative fluorescence and phospholipid phosphorus content of microsomes were essentially identical. The change in the emission maximum during the experiment was never more than 5 nm (475 to 470 nm). The decay curve for ANS-microsome fluorescence produced by phospholipase C is not unlike that reported by Vanderkooi & Martonosi (1969) for sarcoplasmic reticulum, phosphatidylcholine or lysophosphatidylcholine, although the latter authors did not measure the concomitant loss of phospholipid phosphorus.

Fig. 5. Time-course of the effect of phospholipase C on microsomal phospholipid and on the enhancement of ANS fluorescence by microsomes. The solution contained 100 μ M-ANS, microsomes at 1 mg of protein/ml, 0.15 mg of phospholipase C/mg of microsomal protein, 1 mM- CaCl_2 in 0.1 M-HEPES buffer at pH 7.4. Excitation wavelength was 378 nm and the relative intensity of fluorescence was read at 475 nm. ●, Relative fluorescence; ○, microsomal phospholipid. The temperature was 25°C.

Interaction of ANS with phospholipid micelles. The fluorescence of ANS was enhanced by phosphatidylcholine micelles in a manner similar to that obtained with hepatic microsomal membranes.

Fig. 6. Relative fluorescence of ANS in combination with micellar dispersions of phospholipids. Δ , ANS; $\circ$, ANS plus 1 mM-(+)-benzphetamine; $\bullet$, ANS plus 10 mM-warfarin. (a) (Distearoyl) L- α -phosphatidylcholine; (b) (dioleoyl) L- α -phosphatidylcholine; (c) bovine phosphatidylcholine; (d) phosphatidic acid (sodium salt). Phospholipids were at a concentration of 0.25 mg/ml in 50 mM-phosphate buffer, pH 7.4. Relative fluorescence was read at the maximum of the emission spectra (470–490 nm).

Emission maxima were between 475 and 500 nm. The magnitude of the enhancement appeared to be a function of the phosphatidylcholine fatty acid composition (Fig. 6). Unsaturated (dioleoyl) L- α -phosphatidylcholine enhanced ANS fluorescence much more than an equimolar concentration of saturated (distearoyl) L- α -phosphatidylcholine; bovine phosphatidylcholine, which contains both saturated and unsaturated fatty acids, was intermediate in the magnitude of enhancement. A micellar dispersion of phosphatidic acid, which does not contain a choline moiety, gave only a small enhancement of ANS fluorescence. (+)-Benzphetamine significantly enhanced the fluorescence for all phospholipids studied. At low ANS concentrations, ANS-phosphatidylcholine fluorescence was decreased slightly by the anionic drug warfarin (10 mM).

Effects of trypsin treatment of microsomes on ANS

fluorescence and drug metabolism. Digestion of rat hepatic microsomes with trypsin (EC 3.4.4.4) produced a 25% loss of microsomal protein. Continued incubation or the addition of fresh enzyme after 30 min failed to yield further proteolysis. As reported by Orrenius, Berg & Ernster (1969), cytochrome b_5 was released from the membrane and cytochrome P-450 was converted into cytochrome P-420. Microsomal metabolism of (+)-benzphetamine or aniline was completely obliterated. Trypsin-treated microsomes did not exhibit type I or type II difference spectra. The fluorescent properties of ANS bound to trypsin-treated microsomes was the same as that obtained with control microsomes. Neither the emission spectrum nor the fluorescent-enhancement factor of ANS was altered. Drug modulation of ANS-microsome fluorescence as reported above, was unaffected by trypsin treatment.

DISCUSSION

The interaction of the fluorescent probe ANS with hepatic microsomal preparations and micellar dispersions of phospholipids is characterized by an increased quantum yield of fluorescence and a shift of the maximum emission to shorter wavelengths (blue shift). These properties are also observed for ANS and other related fluorochrome naphthalene compounds when they are placed in solvents of lower polarity (Stryer, 1965; Edelman & McClure, 1968). The effects of other environmental factors such as pH or solvent viscosity are less significant. These findings suggest that ANS is a hydrophobic probe, i.e. quantitative and qualitative changes in ANS fluorescence are responses to changes in the polarity of the solvent environment. On these grounds it is reasonable to assume that the fluorochrome is bound or partitioned into a hydrophobic phase when this compound interacts with microsomes and synthetic or isolated phospholipids.

There are several findings to suggest that ANS-microsome fluorescence is largely attributable to ANS binding to membrane phospholipids. (1) Digestion of microsomes with phospholipase C or D markedly decreased ANS fluorescence. (2) Micellar dispersions of phospholipids modified ANS fluorescence in a manner similar to that observed with microsomes. (3) The treatment of selected phospholipid micelles with phospholipase C drastically decreased ANS-phospholipid fluorescence (Vanderkooi & Martonosi, 1969). (4) ANS-microsome fluorescence appeared to be directly related to microsomal content. (5) Modification of ANS-microsome and ANS-phospholipid fluorescence by drugs was similar. (6) Trypsin treatment did not alter ANS-microsome fluorescence.

Phospholipase C (*Cl. welchii*) was reported by Martonosi *et al.* (1968) to degrade mainly phosphatidylcholine of muscle sarcoplasmic reticulum. Phospholipids such as phosphatidylserine and phosphatidylethanolamine are less susceptible to hydrolysis by phospholipase C. Tzur & Shapiro (1969) found this also to be true for rat hepatic microsomal preparations. Our value for residual phospholipid phosphorus retained in microsomes after phospholipase C treatment is in agreement with the latter workers.

After removal of phospholipid from microsomes, the overall vesicular and trilaminar structure is preserved. Trump, Duttera, Byrne & Arstila (1970) found that the diglycerides formed by phospholipid degradation are retained as 'droplets' in association with the interior of the microsomal membrane. Phosphorylcholine, also formed by the action of phospholipase C on microsomes, is liberated from the membrane owing to its water solubility. The

lipid-soluble product of phospholipase D, phosphatidic acid, is also retained in a functionally active form by the membrane (Martonosi *et al.* 1968). Lenard & Singer (1968) treated erythrocyte membranes with phospholipase C and found that the membrane remains intact under phase-contrast microscopy; the average protein conformation in the membranes, as determined by circular-dichroism measurements, is unaffected. However, Trump *et al.* (1970), using hepatic microsomes, concluded that modification of membrane phospholipid by phospholipase C is associated with 'widening' of the membrane which is due to changes in protein conformation; this effect can be reversed by reconstitution with phospholipid.

N.m.r. studies (Chapman, Kamat, DeGier & Penkett, 1968) indicate that the choline $[(CH_3)_3N^+]$ groups of membrane phosphatidylcholine might have considerable freedom of movement; movement of the acyl moieties, or the hydrophobic tails, of the phospholipids was found to be severely restricted, possibly by extensive hydrophobic bonding with membrane proteins. These results support the membrane model (Wallach & Zahler, 1966; Vanderkooi & Green, 1970) where the ionic and polar heads of the phospholipids are situated at the exterior surfaces of membranes in contact with the bulk aqueous phase. This would account for the relative ease by which phospholipase C attacks microsomal phosphatidylcholine, as well as the ability of membrane phospholipids to act as ion exchangers to anionic and cationic agents (Blaustein, 1967).

From the above studies, the nature of the binding of ANS to microsomal membranes or synthetic phospholipids may be proposed. As an anionic compound, ANS is attracted electrostatically to the quaternary ammonium head of microsomal phosphatidylcholine. The hydrophobic naphthalene component of ANS would strongly interact by van der Waals forces with the apolar hydrocarbon chains of phosphatidylcholine, or other lipids. When the choline moiety is removed by phospholipase C or D, or when synthetic phosphatidylcholine is replaced by phosphatidic acid, ANS fluorescence is decreased. Phosphatidylethanolamine and phosphatidylserine micellar dispersions give only a mere fraction of the enhancement of ANS fluorescence obtained with lysophosphatidylcholine or phosphatidylcholine (Vanderkooi & Martonosi, 1969). Thus ANS bound to microsomal membranes is acting as a cationic-hydrophobic probe, as was concluded from studies with detergent micelles by Rubalcava *et al.* (1969).

The binding of ANS to microsomal phospholipid as depicted above might account for the high-affinity site of ANS-microsome fluorescence. This site or binding complex appears to have been removed or

masked selectively by treatment with phospholipases or warfarin respectively. Phospholipases C and D removed the cationic binding site, whereas warfarin, as an anionic drug, competed with the probe for the same cationic site. When the cationic head of phosphatidylcholine is removed, as in the case of phospholipase treatment or phosphatidic acid micelles, warfarin inhibition is decreased or totally absent. An adequate explanation of the nature of the low-affinity site for ANS is speculative. It is possible that in accord with a mosaic membrane model a low-affinity site might be ascribed to phospholipids which are in a physical state different from the remainder of the phospholipids and more tightly coupled to membrane proteins (Glaser, Simpkins, Singer, Sheetz & Chan, 1970).

Except for warfarin, all other compounds investigated enhanced ANS-microsome or ANS-phospholipid fluorescence. This enhancement is unlike the fluorescence titration curves obtained with cations (Vanderkooi & Martonosi, 1969), but more similar to first-order kinetics. This enhancement might be ascribed to these compounds making the interior, apolar end of membrane phospholipids more hydrophobic.

That substrates or inhibitors of hepatic microsomal drug-metabolizing enzymes modify ANS-microsome fluorescence might suggest a role for phospholipids in the biotransformation of drugs and other foreign compounds. Such a role is further corroborated by the significant loss of drug oxidase activity upon pretreatment of microsomes with phospholipase C or D. The decrease of microsomal phospholipids produced by phospholipases could modify the active tridimensional structure of drug-metabolizing enzymes, as well as remove an essential drug-binding component. The present report supports earlier observations (Chaplin & Mannering, 1969), where phospholipase C was shown to decrease the microsomal metabolism and spectral extinction of type I substrates, whereas the same measurements for aniline, a type II substrate, were only slightly affected by this pretreatment. Treatment with phospholipase D in the present study decreases aniline microsomal metabolism possibly by making the membrane electronegative through the formation of phosphatidic acid.

It is apparent from these studies that the type I spectral change is phospholipid-dependent and that ANS interacts with the type I site. Thus the enhancement of ANS-microsome fluorescence by (+)-benzphetamine is a consequence of this drug and the ANS probe conjugating at the type I phospholipid site. The microsomal difference spectra produced by aniline and warfarin, both type II ligands, are not modified by the presence of ANS; however, ANS inhibited the absorption changes produced by (+)-benzphetamine, a type I

ligand (DiAugustine *et al.* 1970). This gives further credence to a common microsomal binding site for ANS and type I ligands. The type II ligands, usually nucleophilic, are thought to form a ferrihaemochrome with cytochrome *P*-450 (Schenkman, Remmer & Estabrook, 1967). By the above scheme, type I compounds would interact with the appropriate microsomal phospholipid, from which perturbation of the haem moiety of cytochrome *P*-450 would occur. Coincidentally, Hutterer *et al.* (1970) reported a phospholipase C-like effect in microsomes from cholestatic livers of rats. Aniline hydroxylase was only decreased slightly and the binding of this type II ligand was unimpaired; the metabolism and binding of this type I ligand aminopyrine was greatly decreased. These experiments further support the mutual exclusiveness of the type I and type II interaction. In keeping with such studies is the preparation of an enzyme from rat livers with phospholipase C activity (Heller & Shapiro, 1966).

The proposal of a role for phospholipids in the metabolism of drugs is plausible in the light of what we presently know about the fate of foreign compounds, or xenobiotics, in mammals. Compounds, unless they are lipid-soluble, are not likely to be metabolized to any great extent. Evidently, they cannot penetrate readily into sites where they can be metabolized. In addition, a hepatic microsomal phospholipid acceptor would be in accord with the broad substrate non-specificity of drug oxidations. Schulze & Staudinger (1970) changed the phospholipid content of rat and guinea-pig hepatic microsomes by various pretreatments *in vivo*. They concluded that cytochrome *P*-450 is a lipoprotein from the close correlation between the changes in the phospholipid and cytochrome *P*-450 content. Williamson & O'Donnell (1969) showed a phospholipid enhancement of an 11 β -hydroxylase prepared from an acetone-dried powder of bovine adrenal mitochondria. The stimulatory action of phospholipid on this enzyme was attributed to its effect on cytochrome *P*-450. Further, phospholipid stabilized the haemoprotein, preventing decomposition of cytochrome *P*-450 to cytochrome *P*-420 during incubation. Lu, Strobel & Coon (1970), using a solubilized form of mixed-function oxidase containing cytochrome *P*-450 of rabbit hepatic microsomes, demonstrated an absolute requirement for a heat-stable lipid factor for the hydroxylation of various drugs and alkanes.

Strobel, Lu, Heidema & Coon (1970) identified this heat-stable factor as phosphatidylcholine. Synthetically prepared dioleoylglycerol-3-phosphorylcholine was reported to be fully active when substituted for this factor as judged by the rate of the hydroxylation of (+)-benzphetamine and other substrates. Drugs appear to have a greater affinity for unsaturated phospholipids than their corres-

ponding saturated congeners. This is given support in the present study by the fact that micelles of phospholipids containing unsaturated acyl groups give decidedly greater enhancement of ANS fluorescence with the affinity of (+)-benzphetamine, as reflected by the enhancement of ANS fluorescence, greatest for micelles of dioleoylglycerol-3-phosphorylcholine.

We thank the following for generously providing us with compounds: Dr R. E. McMahon (2,4-dichloro-6-phenylphenoxyethylamine hydrobromide and 2,4-dichloro-6-phenylphenoxyethyl-diethylamine hydrobromide; Lilly Research Laboratories, U.S.A.) and Dr P. W. O'Connell [(+)-benzphetamine; Upjohn Co., U.S.A.]. The excellent technical assistance of Mrs Gladys Rios, Mrs Roberta Pohl and Mrs T. E. Eling is gratefully acknowledged. This work was supported by U.S. Public Health Service Grants GM-12675 and 5T1-TM-00141 from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

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