# Reaction of Chemical Probes with Phosphatidylethanolamine of Liver Microsomes *

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The two main lipids of the microsomal membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The latter makes up about 30 % of the total. Distribution of phospholipids within the membrane has a great significance since many important properties such as permeability, stability and enzyme activity are often dependent on the presence of specific lipids at defined locations.[1] In previous investigations it was found that the microsomal PE is to a large extent localized on the outer, cytoplasmic surface of the microsomes.[2] These experiments, however, were performed using phospholipase $A_2$ (PLase $A_2$) which may perturbate membranes. Therefore, it is possible that the phospholipases not only hydrolyze lipids available on the outer surface of the vesicles but also induce and increase mobility of lipids, thereby secondarily causing molecular rearrangements. We have tested a number of amino-reacting reagents on intact microsomal vesicles in order to avoid this possible problem and to obtain more information on the structure and composition of these membranes.

The reagents used were 1,5-difluoro-2,4-dinitrobenzene (DFDNB), 1-fluoro-2,4-dinitrobenzene (FDNB), fluorescamine (FA), methyl acetimidate (MA), dimethyl 3,3-dithiobispropionimidate (DTBP) and isoethionylacetimidate (IA). The concentrations of the individual probes were analyzed by constructing a concentration curve reaching the plateau values. After the reaction with isolated microsomes, the lipids were extracted with chloroform – methanol (2:1). Following partition, separation of the reacted PE from the non-reacted part was accomplished by thin layer chromatography on silica gel plates using in the first direction chloroform – methanol – water (65:24:4 v/v), and chloroform – acetone – methanol – acetic acid – water (50:20:10:10:5 v/v) in the second direction. After chromatography, the plates were stained with iodine and scraped; then the amount of phosphate was determined.

Depending on the properties of the probes, different reaction patterns with PE were obtained (Table 1). DFDNB is a bifunctional probe which penetrates membranes easily and has previously been used in a number of studies. At a plateau concentration all PE of the microsomes is reacted, which is consistent with earlier studies.[3] The monofunctional variant of this reagent, FDNB, also freely penetrates membranes and, again, reacts with all PE upon incubation. Three other non-charged reagents were also tested for interaction with microsomes.[4] FA is insoluble in water, as opposed to MA and DTBP; FA and MA are monofunctional but DTBP is a bifunctional reagent. It appears that these reagents penetrate most membranes, and, consequently, they should react with all species of microsomal PE. However, at plateau concentrations, of all three reagents, only 70 % of the PE is reacted, indicating that a part of the lipid is in a compartment which is not available for the probes. To further investigate this question, two concentrations of Triton X-100 were applied: 0.05 % which increases membrane permeability for macromolecules and 0.25 % which solubilizes some components. Complete reaction was obtained with MA in the presence of 0.05 % Triton, while the higher Triton concentration was necessary for the reaction of FA with all PE. On the other hand, interaction with DTBP could not be increased even in the presence of high Triton concentration. It appears that a part of the PE is compartmentalized in the membrane and not easily available; the reaction of the probe with this part of the lipid depends on the properties of the probe itself. The charged monofunctional probe, IA, as opposed to

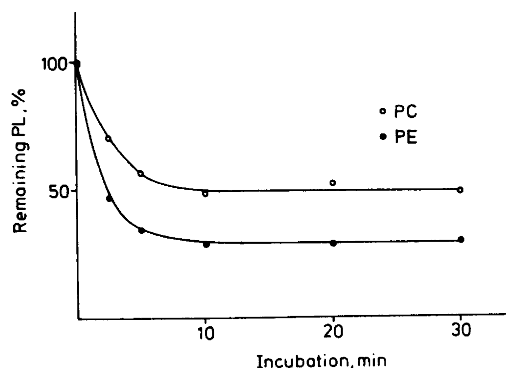


*Fig. 1.* PLase $A_2$ treatment of microsomes. Liver microsomes were incubated with purified PLase $A_2$ (0,5 IU/mg) in the presence of 1 mM $CaCl_2$ and 40 mg bovine serum albumin/ml. Incubations were conducted at 0 °C. The values give the phospholipid remaining in the membranes after centrifugation.

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*Table 1.* Reaction of various amino-reacting probes with microsomal PE. Isolated washed total liver microsomes were incubated in a medium containing 0.1 M phosphate buffer, pH 8.0, 0.25 M sucrose, microsomes and the probe given below. When indicated, Triton X-100 was included in the incubation medium. The incubation was performed at 20 °C for 2 h for DFDNB and FDNB, 1 h for MA and IA and 2 min at 0 °C for FA. The trichloroacid (5 %) precipitated microsomes were extracted with chloroform –methanol 2:1 and the reacted and unreacted PE were separated by thin layer chromatography. Abbreviations see text. The data are given as mean values ±S.E.M. ($n=5$).

| Probe | Concentration µmol/mg protein | Total PE reacted, % None | 0.05 % Triton | 0.25 % Triton |
|---|---|---|---|---|
| DFDNB | 0.05 | 33±3 | | |
| | 0.2 | 98±5 | | |
| | 0.3 | 99±2 | | |
| FDNB | 0.25 | 47±4 | | |
| | 0.75 | 97±6 | | |
| | 1.5 | 98±3 | | |
| FA | 0.5 | 48±6 | | |
| | 1.0 | 68±8 | | |
| | 2.0 | 70±3 | 75±4 | 96±7 |
| MA | 25 | 42±2 | | |
| | 75 | 67±8 | | |
| | 125 | 68±6 | 92±11 | |
| DTBP | 25 | 33±3 | | |
| | 50 | 67±5 | | |
| | 100 | 70±4 | | 75±7 |
| IA | 10 | 6±1 | | |
| | 25 | 13±2 | | |
| | 50 | 14±1 | 16±1 | 51±4 |

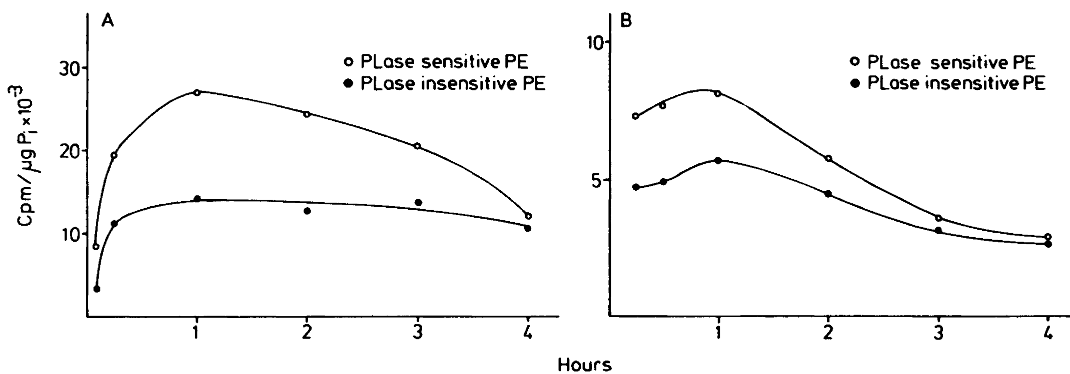


*Fig. 2.* PLase $A_2$ treatment of *in vivo* labeled microsomes. a, Rats were injected with 7.4 MBq [$^3$H]ethanolamine into the portal vein at various time-points before decapitation. The isolated liver microsomes were subjected to PLase $A_2$ treatment (0,5 IU/mg, 0 °C, 15 min) and the specific radioactivity was determined in the PE isolated from the pellet after ultracentrifugation. b, Rats were injected into the portal vein with 7.4 MBq [$^3$H]glycerol and the incubations and measurements were performed as in a.

the other probes, does react badly with microsomal PE, and only a partial interaction takes place even in the presence of high detergent concentrations.

When intact microsomes were treated with PLase $A_2$, the PE could again be divided into two pools (Fig. 1). About 70% of the PE could be removed from the microsomal vesicles. Half of the largest component, PC, was untouched during the hydrolysis.

The metabolic activity of the phospholipid in the PLare $A_2$ sensitive and insensitive compartments is obviously different (Fig. 2). When liver microsomes were prepared from rats *in vivo* labeled with [$^3$H]ethanolamine, the specific activity of the PE in the pool sensitive to PLase $A_2$ was about double that of the lipid in the other pool. The value in the PLase $A_2$-sensitive pool was calculated from the difference between the PE from non-treated microsomes and the PE remaining after enzyme hydrolysis. Equilibration occurred only after 4 h. The situation is quite similar when the *in vivo* labeling is performed with [$^3$H]glycerol.

The experiments with the mono- and bifunctional probes are in agreement with the PLase $A_2$ experiments. Separate from the problem of outside – inside localization, there is obviously a compartmentalization of the PE in the membranes. One is available for probes and hydrolytic enzymes; this part also possesses a high turnover in comparison with the remaining PE. The second pool is probably present for functional reasons and does not only reflect the bilayer distribution. It may be associated with proteins buried in the inner compartments, or the lipid may take part in the formation of inverted micelles.

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