# Microsomal membranes contain phosphatidylcholine that equilibrates across the bilayer, and phosphatidylcholine that does not

Julie L. Hutson, Joan A. Higgins* and K.W.A. Wirtz

*Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, England* and *Biochemistry Laboratory, State University of Utrecht, Transitorium 3, Padualaan 8, 3508 TB Utrecht, The Netherlands*

Received 6 February 1985; revised version received 19 February 1985

Results of experiments using phosphatidylcholine transfer protein and phospholipase C as probes indicate that there are at least two pools of phosphatidylcholine in rat liver microsomes. One of these is preferentially labelled with [$^{14}$C]choline and does not equilibrate across the bilayer. The second pool is labelled with [$^{3}$H]glycerol and does equilibrate across the bilayer. Our observations also confirm that phosphatidylcholine exchange protein does not modify the distribution of phospholipids or cause randomization of the inner and outer leaflet pools of phosphatidylcholine when these are differentially labelled by [$^{14}$C]choline.

*Rat liver microsome* *Asymmetry* *Phosphatidylcholine* *Phospholipase C* *Phosphatidylcholine exchange protein* *Phosphatidylcholine pool*

## 1. INTRODUCTION

Phospholipase C has been used as a probe of the transverse distribution of phospholipids about the bilayer of the endoplasmic reticulum (microsomal fraction) of rat liver. Approx. 50% of the membrane phospholipid was hydrolysed at equilibrium and the vesicles remained intact indicated by retention of labelled secretory protein contents, retention of mannose-6-phosphatase latency and morphology of both sectioned and freeze-fracture preparations in the electron microscope [1–4]. When microsomal vesicles were opened using the French pressure cell, detergents or alkaline pH, so that both sides of the membrane were exposed, hydrolysis of the phospholipids increased up to 80%. On this basis phospholipase C appears to be a valid probe for the transverse distribution of phospholipids in microsomal membranes. Results from experiments using this probe have suggested that microsomal membrane phospholipids are distributed asymmetrically with approx. 70% of the phosphatidylcholine and sphingomyelin and 30% of the phosphatidylethanolamine and phosphatidylserine located in the outer leaflet of the membrane bilayer. A similar distribution of phosphatidylethanolamine was found using trinitrobenzenesulphonate as a probe [5].

[$^{14}$C]Choline injected intraportally or intraperitoneally into rats, before isolation of liver microsomes, or incubated with rat liver microsomes as cytidine diphospho[$^{14}$C]choline in vitro is incorporated preferentially into a pool of phosphatidylcholine which is available for hydrolysis by phospholipase C [6]. It appears, therefore, that phosphatidylcholine synthesised by the cytidine pathway is incorporated into the outer leaflet of the microsomal membrane bilayer. This outer leaflet pool of phosphatidylcholine does not equilibrate with that in the inner leaflet in vivo or in vitro [7]. In contrast, the specific activities of inner and outer leaflet phosphatidylcholine are similar when labelled in vivo with either [$^{3}$H]glycerol or [$^{14}$C]methylmethionine. Therefore,

* To whom correspondence should be addressed

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/85/$3.30 

there are pools of phosphatidylcholine in microsomes which are labelled by different precursors. [$^{14}$C]Choline is incorporated into a pool which is preferentially retained in the outer leaflet while [$^{3}$H]glycerol or [$^{14}$C]methylmethionine are incorporated into pools in the outer and inner leaflet which are apparently in equilibrium.

Phospholipid transfer proteins exchange phospholipids between the outer leaflets of membranes and liposomes [7,8] and have been used successfully in investigations of the transverse distribution of phospholipids of erythrocyte membranes [9,10]. These proteins are attractive probes for membrane phospholipid distribution as they are active under physiological conditions and do not modify membrane structure by chemical labelling or hydrolysis of the phospholipids. However, these transfer proteins have not proved suitable for investigating the transverse distribution of phospholipids in microsomal membranes. Specifically, application of the phosphatidylcholine transfer protein from bovine liver indicated that all of the phospholipids of these membranes are exchanged on incubation of microsomes with excess phospholipid in mitochondria, liposomes or high density lipoproteins [11,13].

The results of the two groups of investigations above are difficult to reconcile. Studies with phosphatidylcholine transfer proteins indicate that phosphatidylcholine moves across the bilayer of the microsomal membrane, so that it is completely exchanged. Investigations using phospholipase C as a probe indicate that phosphatidylcholine synthesised by the cytidine pathway remains in the outer leaflet of the bilayer and does not move to the inner leaflet. One explanation of this discrepancy is that transfer proteins may cause transmembrane movement and randomization of phospholipids in microsomal membranes. To investigate this possibility we have used both phospholipase C and phosphatidylcholine transfer protein to investigate the availability of the different pools of phosphatidylcholine to each probe.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Egg phosphatidylcholine and phosphatidic acid were purchased from Lipid Products, Nutfield, Surrey. Radiolabelled chemicals were purchased from Amersham International; phospholipase C, type XII or type X, was purchased from Sigma.

### 2.2. *Rat liver microsomes*

Rat liver microsomes were prepared as described [14,15]. These were labelled by intraportal injection of [$^{14}$C]choline (5 $\mu$Ci/100 g body wt) or intraperitoneal injection of [$^{3}$H]glycerol (5 $\mu$Ci/100 g body wt) 1 h prior to killing of the rats, removal of the liver and preparation of microsomes.

### 2.3. *Phosphatidylcholine transfer protein*

Phosphatidylcholine transfer protein specific for transfer of phosphatidylcholine was prepared from bovine liver as in [16].

### 2.4. *Liposomes*

Liposomes were prepared from egg phosphatidylcholine with 2% phosphatidic acid. The lipids were dispersed in 0.25 M sucrose, 0.01 M Tris, 0.001 M EDTA, pH 7.4 (SET buffer) at 0°C by sonication using an MSE sonicator at setting 5 for 10 min. The dispersed lipids suspension was centrifuged at 105000 × *g* for 90 min. The upper three quarters of the suspension in the centrifuge tube was removed and used as the liposome preparation.

### 2.5. *Exchange experiments*

In exchange experiments radiolabelled microsomes were incubated with excess phospholipids dispersed as liposomes in 1 ml SET buffer with and without transfer protein at 25°C for a range of times. At the end of the incubation period the microsomal suspension was layered onto 9 ml of 0.75 M sucrose in centrifuge tubes and centrifuged at 105000 × *g* for 90 min. The microsomes formed a pellet and the liposomes remained in the load layer. The liposomes were removed and the lipids extracted by the method of Bligh and Dyer [17]. The supernatant was removed carefully, the microsomal pellet resuspended in a small volume of buffer and the lipids extracted with chloroform/methanol/HCl (2:1:0.02, by vol.) as described [6]. Aliquots of the lipid extracts were separated into phospholipid classes and the specific activities of the phosphatidylcholine determined as in [6].

In preliminary experiments liposomes were labelled by addition of a trace amount of

[$^{14}$C]cholesteryl oleate to the lipids prior to sonication and the microsomal proteins were labelled with [$^{3}$H]leucine [18]. When these were mixed without transfer protein and separated as described above, the cross contamination between the separated fractions indicated by $^{14}$C in the microsomal pellet and $^{3}$H in the liposomes was less than 5%. In all experiments recovery of the microsomal protein and phospholipid and the liposomal phospholipid was greater than 90% and the microsomes retained their integrity indicated by retention of mannose-6-phosphatase latency [19].

### 2.6. *Treatment with phospholipase C*

In experiments in which microsomes were treated with phospholipase C after exchange with transfer protein the microsomal pellets were resuspended in 0.15 M NaCl containing 1 mM $CaCl_2$ and adjusted to pH 7.4 with bicarbonate to give a concentration of 5 mg protein/ml. Aliquots (1.0 or 0.5 ml) were incubated with and without phospholipase C, 10 units/ml, for 5 min at 37°C. Under these conditions hydrolysis of microsomal phospholipids reached a plateau within 2 min incubation with approx. 50% hydrolysis. The reaction was stopped by addition of EDTA (10 mM) and the lipids were extracted and analysed as above.

## 3. RESULTS AND DISCUSSION

When microsomes labelled with phosphatidyl-[$^{14}$C]choline were incubated with liposomes in the presence of phosphatidylcholine transfer protein the labelled phospholipid rapidly exchanged with that of the liposomes. Equilibrium was reached after 1 h with approx. 63% of the labelled phospholipid lost from the microsomes (fig.1). In the absence of transfer protein there was less than 10% transfer of label into the liposomal fraction. If two thirds of the liposomal phosphatidylcholine [20] and all of the microsomal phosphatidylcholine is available for exchange it can be calculated [13] that equilibrium would be reached at 68% of the labelled phosphatidylcholine transferred to the liposomes. If 70% of the microsomal phosphatidylcholine were available [1] this figure would be 77%. These observations suggest therefore that all of the microsomal phosphatidylcholine is available for exchange. On the other hand, previous studies using phospholipase C have indicated that 70% of the microsomal phosphatidylcholine is in the outer leaflet of the microsomal membrane bilayer and that [$^{14}$C]choline is preferentially incorporated (i.e., 90% of the total label) into this pool [6]. As described previously [6], the specific activity of the

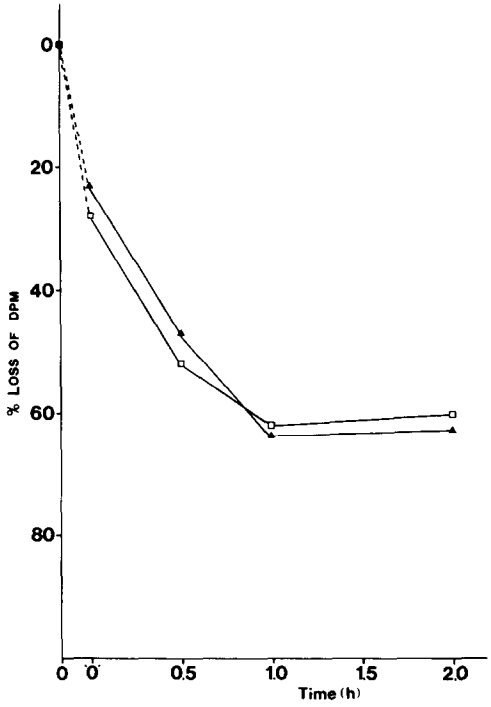


Fig.1. Percent exchange of phosphatidylcholine between liposomes and microsomes catalysed by phosphatidylcholine transfer proteins. Microsomes in aliquots equivalent to 0.65 mg phosphatidylcholine were incubated with liposomes containing 2.25 mg phosphatidylcholine in 1 ml SET buffer, with 0.05 ml phosphatidylcholine transfer protein at 25°C. At each time point samples were separated into liposomes and microsomes and the lipids extracted and analysed as described in section 2. The first time point indicates the radioactivity of the microsomes prior to incubation. The zero time point indicates the exchange which occurs when microsomes are mixed with liposomes in the presence of phosphatidylcholine transfer protein and immediately separated by centrifugation. Other time points indicate the time of incubation at 25°C before separation of the microsomes and liposomes by centrifugation. The loss of radioactivity from phosphatidylcholine labelled by [$^{14}$C]choline (□—□) or [$^{3}$H]glycerol (▲—▲) is plotted vs time.

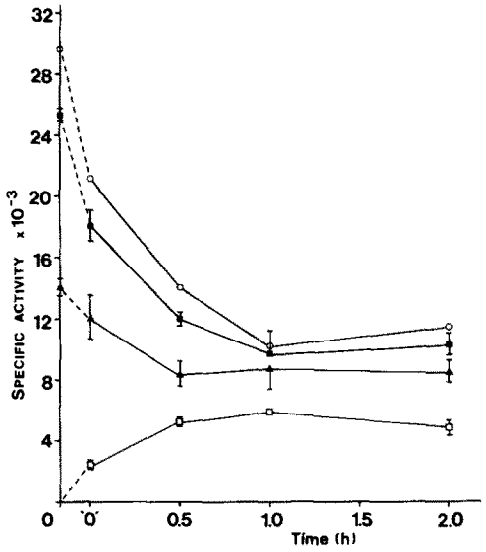


Fig.2. Specific activities of phosphatidylcholine labelled by [$^{14}$C]choline of the inner and outer leaflets of the microsomal membrane after exchange by phosphatidylcholine transfer protein. Incubation conditions and experimental procedures were as in fig.1. The specific activities of the total phosphatidylcholine of microsomes and the liposomes, the phosphatidylcholine not hydrolysed by phospholipase C (inner leaflet) and the calculated specific activities of the phosphatidylcholine hydrolysed by phospholipase C (outer leaflet) are plotted vs time. Each point is the mean of 4 determinations; the standard deviations are indicated by bars. Specific activities of phosphatidylcholine of liposomes (□—□); of total microsomes (■—■); of inner leaflet (▲—▲) and of outer leaflet (○—○).

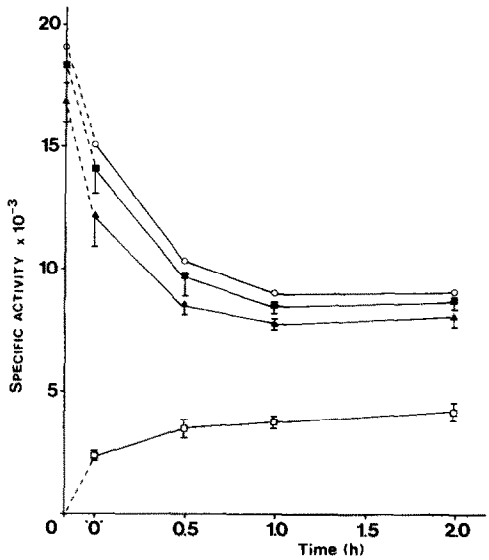


Fig.3. Specific activities of phosphatidylcholine labelled by [$^{3}$H]glycerol of the inner and outer leaflets of the microsomal membrane after exchange by phosphatidylcholine transfer protein. Experimental details as in fig.2 except that microsomes were labelled with [$^{3}$H]glycerol.

pool of phosphatidylcholine available for hydrolysis by phospholipase C (outer leaflet) was greater than that remaining unhydrolysed (inner leaflet) by a factor of more than 2.0. The specific activities of both pools of phosphatidylcholine fell on incubation with liposomes and transfer proteins (fig.2) indicating that both leaflets of the membrane bilayer participate in the exchange reaction. That at equilibrium the specific activity of the inner leaflet phosphatidylcholine was still less than that of the outer leaflet, suggests that these phosphatidylcholine pools do not equilibrate completely during exchange.

Similar experiments were also performed in which the microsomal phospholipids were labelled with [$^{3}$H]glycerol. This precursor is incorporated into all of the phospholipids of the microsomal membrane. As shown in fig.3 the specific activities of the phosphatidylcholine pools of the inner and outer leaflets of the membrane are closer than with [$^{14}$C]choline as a label. Exchange of phosphatidylcholine labelled with [$^{3}$H]glycerol was rapid and reached equilibrium when 64% of the labelled phospholipid was lost from the microsomes (fig.1). This agrees with the model that all of the microsomal phosphatidylcholine is available for exchange with approximately two thirds of the liposomal phospholipid. The specific activities of phosphatidylcholine in the inner and outer leaflet of the microsomal membrane fell at the same rate to reach equilibrium with the liposomes after 60 min incubation (fig.3).

The above observations are consistent with previous reports that phosphatidylcholine transfer protein catalyses complete exchange of phosphatidylcholine of microsomal membranes. If this interpretation is correct, it is hard to understand how two pools of phospholipid can still be distinguished by phospholipase C after exchange has taken place (fig.2). One possible explanation of these results is that the transfer protein causes local

Table 1

Specific activities of phosphatidylcholine of the inner and outer leaflets of the microsomal membrane after exchange by phosphatidylcholine transfer protein

| | % hydrolysis of total phospholipid | % hydrolysis of labelled phospholipid | Total | Specific activities of phosphatidylcholine (dpm/μmol) | |
|---|---|---|---|---|---|
| | | | | Inner leaflet | Outer leaflet |
| PTP treated | 52 | 90 | 5231 + 81 | 2343 + 81 | 6675 |
| Incubated without PTP | 48 | 92 | 5340 + 6 | 2793 + 349 | 6614 |

Rat liver microsomes labelled with [$^{14}$C]choline were incubated with and without phosphatidylcholine transfer protein without liposomes as described in section 2 for 2 h. The microsomes were isolated by centrifugation and treated with phospholipase C as described in section 2. The specific activities of the phosphatidylcholine of the total microsomal phospholipid, and that of the inner leaflet (not hydrolysed by phospholipase C) are given as averages of 4 determinations ± SD. The specific activities of the outer leaflet phosphatidylcholine (hydrolysed by phospholipase C) were calculated from the average specific activities of the hydrolysed and total pools [6]

transient randomization of phosphatidylcholine in the bilayer during the formation of the transfer protein-membrane collision complex. To investigate this possibility we prepared microsomes labelled with [$^{14}$C]choline in which the specific activity of the outer leaflet of the bilayer was more than twice that of the inner leaflet (table 1). These were incubated with excess transfer protein in the absence of liposomes to allow complete exchange with the microsomal vesicle population, and the specific activities of the two pools of phosphatidylcholine determined using phospholipase C. Under these conditions the specific activities of the phosphatidylcholine of the inner and outer leaflets of the membrane were unchanged. The transfer protein does not, therefore, cause randomization of the two pools of phosphatidylcholine. This implies that the observed exchange of the inner leaflet phosphatidylcholine is due to transmembrane exchange of phospholipid with that of the outer leaflet.

These observations indicate that phosphatidylcholine of microsomal membranes exists in at least two pools, which are not in equilibrium. [$^{14}$C]-Choline apparently labels a pool which does not equilibrate across the bilayer, while [$^{3}$H]glycerol labels a pool which does equilibrate across the bilayer. At present we do not know the relative sizes of the pools nor the reason for their separation. However, it is possible that [$^{14}$C]choline labels two subpopulations of microsomal vesicles: in one subpopulation phosphatidylcholine does equilibrate across the bilayer and in the other it does not. Such a morphological separation would prevent equilibration between the phosphatidylcholine of inner and outer leaflets during exchange with transfer proteins.

## ACKNOWLEDGEMENTS

This research was supported by grants from the Medical Research Council of Great Britain and the Wellcome Trust.

## REFERENCES

[1] Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342–356.
[2] Bollen, I.C. and Higgins, J.A. (1980) Biochem. J. 189, 475–480.
[3] Hutson, J.L. and Higgins, J.A. (1982) Biochim. Biophys. Acta 687, 247–256.
[4] Higgins, J.A. (1982) J. Cell Sci. 53, 211–225.
[5] Higgins, J.A. and Pigott, C.A. (1982) Biochim. Biophys. Acta 693, 151–158.
[6] Higgins, J.A. (1979) Biochim. Biophys. Acta 558, 48–57.
[7] Zilversmit, D.B. and Hughes, M.E. (1976) Methods Membrane Biol. 7, 211–259.

[8] Wirtz, K.W.A. (1982) in: Lipid-Protein Interactions (Host, P.C. and Griffiths, O.H. eds) vol.1, pp.151–231, Wiley, New York.
[9] Crain, R.C. and Zilversmit, D.B. (1980) Biochemistry 19, 1440–1447.
[10] Van Meer, G., Poorthuis, B.J.H.M., Wirtz, K.W.A., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1980) Eur. J. Biochem. 103, 283–288.
[11] Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99–110.
[12] Van den Besselaar, A.M.H.P., De Kruijff, B., Van den Bosch, M. and Van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242–255.
[13] Jackson, R.L., Westerman, J. and Wirtz, K.W.A. (1978) FEBS Lett. 94, 38–42.
[14] Higgins, J.A. (1976) J. Cell Sci. 22, 173–198.
[15] Higgins, J.A. (1981) Biochim. Biophys. Acta 640, 1–15.
[16] Westerman, J., Kamp, H.H. and Wirtz, K.W.A. (1983) Methods Enzymol. 98, 581–586.
[17] Blight, E.G. and Dyer, W. (1959) Can. J. Biochem. Physiol. 37, 291.
[18] Kreibich, G., Debey, P. and Sabatini, D.D. (1973) J. Cell Biol. 58, 463–472.
[19] Arion, W.J., Ballas, L.M., Lange, A.J. and Wallin, B.K. (1976) J. Biol. Chem. 251, 4901–4907.
[20] Scherphof, G., Van Leeuwen, B., Wilschut, J. and Damen, J. (1983) Biochim. Biophys. Acta 732, 595–599.