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# Transbilayer movement and distribution of spin-labelled phospholipids in the inner mitochondrial membrane

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**Abstract**

The transmembrane diffusion and equilibrium distribution of spin-labelled phosphatidylethanolamine (PE*), phosphatidylcholine (PC*) and cardiolipin (CL*) were investigated in purified mitochondrial inner membranes using electron spin resonance spectroscopy. Using the back exchange technique, we found that the outside–inside movement of PE* and PC* in beef-heart inner mitochondrial membranes was rapid ($t_{1/2}$ in the range 10–15 min at 30°C). The steady-state distributions in non-energised mitoplasts were approximately 30% in the inner leaflet for PC* and 39% for PE*. Within the limits of probe concentration that can possibly be used in these experiments, the initial velocity of the inward movement was not saturable with respect to the amount of analogue added to the membranes, suggesting that the spin-labelled phospholipids diffused passively between the two leaflets of the inner mitochondrial membrane. In energised mitoplasts, PC* behaviour was not affected, PE* diffused approximately two times faster toward the inner monolayer but reached the same plateau. Treatment of energised mitochondria with *N*-ethylmaleimide did not affect PC* diffusion, while the kinetics of PE* internalisation became identical to that of PC*. Similar results were found when PC* and PE* movements were studied in mitoplasts from beef heart, rat liver or yeast. The spin-labelled cardiolipin, which possesses four long chains, had to be introduced in the mitoplast with some ethanol. After equilibration ($t_{1/2}$ of the order of 13 min at 30°C), the transmembrane distribution suggested that approximately half of the cardiolipin analogue remained in the outer leaflet. These results do not allow us to determine if a specific protein (or flippase) is involved in the phospholipid transmembrane traffic within inner mitochondrial membranes, but they show that lipids can rapidly flip through the mitochondrial membrane. 

*Keywords:* Mitochondrion; Lipid asymmetry; Electron spin resonance; Spin label; Cardiolipin

## 1. Introduction

During the last 20 years, a great effort has been given to the investigation of lipid topology and transmembrane lipid diffusion in the plasma membrane of animal cells. The best documented system is the human erythrocyte membrane for which several inde-

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pendent methods have shown that phospholipids are asymmetrically distributed [1]. Phosphatidylcholine (PC) and sphingomyelin (SM) are essentially located in the outer leaflet while aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are present in majority or almost exclusively in the inner leaflet of the plasma membrane [2,3]. Phosphoinositides are also in the majority confined to the cytoplasmic leaflet [4]. Other studies have demonstrated that all mammalian cells have asymmetrical plasma membranes with a lipid distribution comparable to that of human erythrocytes [1,5–8]. It has been established that the accumulation of PS and PE in the inner leaflet of the erythrocyte membrane and of the plasma membrane of other eukaryotic cells is due to the activity of a selective lipid pump, the aminophospholipid translocase [9]. This protein transports aminophospholipids from the outer to the inner monolayer at the expense of hydrolysis of cytosolic ATP and is able to maintain PS and PE asymmetry.

Fewer studies dealt with lipid distribution in organelle membranes where the transmembrane lipid distribution is not so well established. Rapid transmembrane diffusion of phospholipids was shown in the endoplasmic reticulum and the involvement of an ATP-independent 'flippase' is likely although no corresponding protein has yet been purified [10–13]. The transverse diffusion of phospholipids as well as glycolipids was shown to be rapid in purified Golgi fractions [14]. In bovine chromaffin granules, an ATP-dependent transporter has been identified [15,16]. Several publications indicate lipid asymmetry in mitochondrial membranes (see the review by Daum [17]). According to Hovius et al. [18] the outer membrane of rat liver mitochondria has an asymmetric phospholipid distribution with 55%, 77%, 100% and 30% of PC, PE, CL and (PI+PS), respectively, in the outer leaflet. Recently, Dolis et al. [19] determined PC distribution using radio labelled phospholipid introduced in mitochondrial outer membrane vesicles by using PC-specific transfer protein. They found that PC was equally distributed in both leaflets and that the $t_{1/2}$ of the transmembrane movement at 30°C was 2 min or less. This rapid diffusion could be explained by the existence of a phospholipid flippase in the outer mitochondrial membrane or of non-bilayer structures. From $^{31}$P-NMR studies, the occurrence of non-bilayer lipid structures in the inner mitochondrial membrane was reported to be possible [20].

Studies on phospholipid distribution in the inner membrane were carried out on different types of membrane preparations, i.e., right-side-out oriented (mitoplasts) and inside-out oriented (submitochondrial particles) membranes. The results obtained differed according to the origin of the mitochondria (liver, heart) and the reported distributions of lipids in the same membrane were sometimes contradictory. From studies involving long-chain spin-labelled phosphatidylcholine analogues introduced in mitochondrial inner membranes from rat liver, Rousselet et al. [21] concluded in 1976 that the transverse diffusion of PC was extremely slow in such membranes, with a $t_{1/2}$ exceeding several hours at 25°C. The latter result appears now relatively surprising in view of the much more rapid flip-flop found in the outer mitochondrial membrane as well as in most intracellular membranes. Thus, one of the goals of this article is to re-examine phospholipid diffusion in inner mitochondrial membranes.

In the present study, using spin-labelled phospholipid analogues, we investigated PE, PC and cardiolipin (CL) distributions and flip-flop in the inner membrane of mitochondria from different origins. The transmembrane movements of PC and PE spin labels were studied in terms of kinetics, energisation requirement, protein involvement and sensitivity to inhibitors.

## 2. Materials and methods

### 2.1. Preparation of beef-heart, rat-liver and yeast mitochondria and mitoplasts

Beef-heart mitochondria were prepared by differential centrifugation [22]. Briefly, a beef heart was minced in a medium containing 270 mM sucrose, 10 mM Tris–HCl (pH 7.3). The homogenate was centrifuged at $2500\times g$ for 10 min and the pellet was discarded. The pH of the supernatant was adjusted if necessary to 7.3 with 1 M Tris-base. The mixture was centrifuged at $10\,000\times g$ during 15 min. Mitochondria, present in the pellet, were washed twice then resuspended in the same buffer.

Mitoplasts were prepared by two different methods. In protocol A, mitochondria were treated by digitonin as described by Krebs et al. [23]. To a mitochondria suspension in 270 mM sucrose, 10 mM Tris–HCl (pH 7.3), crystallised digitonin (Sigma) was added to the concentration of 0.18 mg digitonin/mg protein. After incubation on ice for 5 min, 10 volumes of 270 mM sucrose, 10 mM Tris–HCl (pH 7.3) were added to dilute digitonin. The mixture was then centrifuged (12 000 × *g*, 10 min) and mitoplasts were gently resuspended in a small volume of the same medium. In protocol B, beef-heart mitoplasts were prepared by freezing mitochondria in liquid nitrogen and thawing as described by Brandolin et al. [24]. Mitoplasts were then washed as above. The orientation of mitoplasts was controlled by the binding of radiolabelled atractyloside, a specific inhibitor of the ADP/ATP carrier which recognises the moiety of the translocator exposed on the outer face of the inner membrane [24].

Rat-liver mitochondria were prepared by differential centrifugation of a liver homogenate in 250 mM sucrose, 2 mM Tris–HCl buffer (pH 7.4) as previously described [25]. Following this procedure, contamination by microsomal membranes is low [25]. Mitoplasts were prepared by digitonin treatment according to the procedure described by Hovius et al. [26].

Mitochondria from yeast grown with lactate as a carbon source were isolated as previously described by Gallet et al. [27]. Mitoplasts were prepared by treatment of mitochondria with digitonin according to the procedure of Velours et al. [28]. Contamination by microsomal membranes was determined by assaying enzymatic marker activities (NADPH–cytochrome *c* oxidoreductase and acid phosphatase). Less than 6% of these activities was found associated with the mitochondrial fraction (P.F. Gallet, unpublished results).

Integrity of beef-heart and rat-liver mitoplasts was assessed by measuring the activity of the matricial enzyme fumarase [29]. The inner mitochondrial membrane remained intact since near 80% of the fumarase activity present in intact mitochondria was recovered in the mitoplast fraction. The elimination of the outer membrane was followed by assaying monoamine oxidase [30]: only 2% and 18% of the monoamine oxidase activity, initially present in mitochondria, remained in the mitoplast pellet from rat liver and beef heart, respectively. The fact that intact mitoplasts could be obtained from beef-heart mitochondria indicated that contamination of mitochondria by lighter membranes was negligible [23].

For yeast mitoplasts, the kynurenine hydroxylase [31] activity was measured instead of monoamine oxidase. After digitonin treatment, less than 15% of the initial kynurenine hydroxylase was found in the mitoplast fraction. On the other hand, 80% of fumarase activity exhibited by intact mitochondria was recovered in the mitoplasts.

### 2.2. Translocation assay for spin-labelled PE and PC

1-Palmitoyl-2-(4-doxyl-pentanoyl)-*sn*-glycero-3-phosphocholine (PC*) and ethanolamine (PE*) were synthesised as previously described [32]. To label mitochondria membranes (4 mg protein $ml^{-1}$), the desired amount (routinely 20 nmol $ml^{-1}$) of PE* or PC* in chloroform solution was dried under reduced pressure. The dried lipid film was resuspended in buffer by vigorous vortexing and then transferred onto the mitoplast suspension. To assess the complete incorporation of the spin labels into inner membranes, an aliquot of membrane suspension was centrifuged 30 s after spin-label addition. The amount of analogue present in the supernatant accounted for less than 5% of the amount present in the suspension. At different times of incubation, 90-μl aliquots were withdrawn and stored on ice for 1 min to reduce the movement of lipids within the membrane. Phospholipid analogues in the outer monolayer were extracted by addition of 25 μl of ice-cold 5% (w/v) fatty acid-free BSA solution in buffer. The mixture was incubated for 2 min and then centrifuged (10 000 × *g* for 2 min at 4°C, Sigma centrifuge). The supernatant (90 μl) was collected and reoxidised by 10 μl of 100 mM potassium ferricyanide before electron spin resonance (ESR) measurement [32].

### 2.3. Translocation assay for spin-labelled CL

Cardiolipin analogue (CL*) was synthesised by reacting 5-doxyl palmitic acid with monolyso cardiolipin (Avanti Polar) [33]. The lyso derivative possesses two sites of esterification leading to a mixture of paramagnetic products. The lipid of interest was iso-

lated by preparative thin-layer chromatography. Its structure was attested by a ratio of phosphorus to spin label close to 2 and by the positive response to phospholipase C treatment.

Due to the presence of four long fatty acyl chains, CL* cannot form micelles as do PC* and PE* and therefore another labelling procedure had to be used. CL* was solubilised in ethanol and injected in mitoplast suspension. In all cases, the ethanol volume never exceeded 1% of the total volume and the CL* added corresponded to 1% of the mitochondrial lipids. However, not all CL* was incorporated in the bilayer and it was necessary to centrifuge the suspension to eliminate the unincorporated analogue. One minute after CL* addition, the mitoplast suspension was diluted 5 times with buffer and centrifuged at $15\,000 \times g$ for 10 min. The pellet was resuspended in a volume equal to the initial one and incubated at 30°C. CL* cannot be extracted from the bilayer by addition of BSA and its distribution in the membrane has to be determined by reduction of the probe following ascorbate addition [21,34]. One hundred-μl aliquots were removed at different incubation times and centrifuged at $15\,000 \times g$ for 2 min at 4°C (Sigma centrifuge). The pellet was resuspended in 90 μl of cold buffer with ascorbate. The ESR signal amplitude of the various aliquots, recorded at 4°C for at least 20 min, decreased with time with a characteristic biexponential function. Very likely the fast component corresponded to the reduction of the probes located in the outer leaflet and the slow component to the reduction of the nitroxides in the inner monolayer. The time required for labelling of the membranes, removal of the unincorporated analogue and reduction by ascorbate of the CL* present in the outer membrane leaflet made it impossible to determine CL* distribution rapidly. The first point corresponded to an elapsed time of 30 min.

### 2.4. Protein and phospholipid determination

The protein content was determined by the Biuret method using crystalline BSA as a standard [35]. Phospholipids were quantified by measuring the lipid phosphorus content using the method of Zhou and Arthur [36] after phospholipid extraction into organic solvents.

## 3. Results

### 3.1. Transmembrane diffusion and equilibrium distribution of PC* and PE* at 30°C

When beef-heart mitoplasts were incubated above 30°C, the ESR signal decreased rapidly in a partially irreversible manner. Thus, in the following experiments spin-label movements were studied at 30°C or below. The outside–inside movement of PE* and PC* was studied with mitoplasts incubated at 30°C in an energising medium (with 20 mM succinate) or a non-energising medium (without succinate). Both probes flipped rapidly from the outer to the inner monolayer. In the case of PE*, the half-time of equilibration was faster in the energised medium ($t_{1/2} \sim 6.5$ min) than in the non-energised medium ($t_{1/2} \sim 15$ min; Table 1). However, the PE* plateau corresponded to approximately 40% of the probe on

Table 1
Characteristics of PE* and PC* reorientation in the membrane of the beef-heart mitoplasts

| | Non-energised | Energised | Energised+1 mM NEM | Energised+5 mM NEM |
|---|---|---|---|---|
| PE | | | | |
| Inside (%) | 39.3 ± 1.1 | 41.5 ± 1.7 | 27.0 | 26.0 |
| Initial rate (%/min) | 1.82 ± 0.45 | 4.20 ± 0.56 | 1.45 | 1.85 |
| PC | | | | |
| Inside (%) | 30.2 ± 1.0 | 30.6 ± 2.4 | 27.7 | 29.7 |
| Initial rate (%/min) | 1.70 ± 0.03 | 2.11 ± 0.38 | 1.56 | 1.72 |

Different conditions have been used: incubation in a saline buffer (non-energised state), energisation by incubation in a succinate-containing medium, treatment by 1 and 5 mM NEM of energised mitoplasts. Values were obtained from the fitting of curves as shown in Fig. 1. See Section 2 for details.
Values are mean of five experiments (± S.D.) except for the studies with NEM.

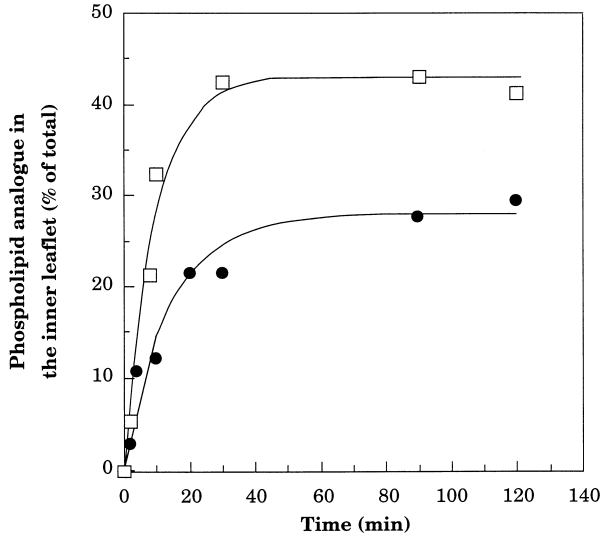


Fig. 1. Kinetics of transmembrane translocation of PC* and PE* in beef-heart mitochondria inner membranes. Mitochondria inner membranes were incubated in a 250 mM sucrose, 2 mM Tris–HCl buffer (pH 7.3) containing 20 mM succinate (energy-rich medium): (●) PC*, (□) PE*. Curves were fitted according to the equation $A(t) = A_{eq}(1-\exp(-kt))$ where $A(t)$: amount of analogue present in the inner leaflet at time $t$; $A_{eq}$: amount of analogue present in the inner leaflet at steady state. Data from a typical experiment are shown.

the inner leaflet in the presence or in the absence of succinate (Fig. 1, Table 1). The translocation of PC* was slightly more rapid in the energised state ($t_{1/2} \sim 10.6$ min) than in the non-energised one ($t_{1/2} \sim 12.5$ min). At equilibrium PC* was preferentially located on the outer leaflet ($\sim 70\%$) (Fig. 1, Table 1).

The movements and steady-state distributions of the two analogues PE* and PC* were also studied, under the energised state, in rat-liver and yeast mitoplasts. As shown in Table 2, no large differences appeared in PC* distribution while PE* seemed more asymmetrically distributed in rat-liver and yeast inner membranes compared to that of beef heart.

### 3.2. Effect of temperature

Beef-heart mitoplasts were incubated in succinate containing medium in the presence of PE* or PC* at 4°C, 15°C and 30°C and the rates of translocation (initial slopes of the kinetic curves) were determined (Fig. 2). For both lipid probes, initial velocities were 8-fold faster at 30°C than at 4°C, PE* translocation being always approximately 2-fold faster than that of PC*. The equilibrium distribution of PC* and PE* appeared insensitive to the temperature. Activation energies for PC* and PE* translocation were comprised between 49 and 53 kJ $mol^{-1}$ $K^{-1}$.

### 3.3. PE* and PC* diffusion as a function of analogue concentration

In the non-energised state, PE* translocation rate, expressed as nmoles per min, varied linearly with the amount of analogue used (Fig. 3). Similar results were obtained with PC* (data not shown). Increasing the concentration from 2.5 to 10 nmol/mg protein did not modify the steady-state distribution nor the translocation velocity in the energised state. Higher concentrations of spin labels cannot be used because of the detergent character of the probes and because membranes with highly asymmetrical lipid ratios would bend and eventually collapse due to the surface tension generated by the mismatch between both monolayers [37].

### 3.4. Effect of sulfhydryl reagents

The sensitivity of PC* and PE* movements to a treatment of the mitoplasts by *N*-ethylmaleimide

Table 2
PE* and PC* reorientations in mitoplasts from beef heart, rat liver and yeast

| | PE* | | PC* | |
|---|---|---|---|---|
| | Inside (%) | Initial rate (%/min) | Inside (%) | Initial rate (%/min) |
| Beef-heart mitoplasts | 41.5 | 4.20 | 30.6 | 2.11 |
| Rat-liver mitoplasts | 32.0 | 3.10 | 28.5 | 1.70 |
| Yeast mitoplasts | 35.0 | 3.14 | 28.0 | 1.51 |

The distribution of phospholipids has been determined by the technique of extraction with BSA. The incubation temperature was 30°C for beef-heart and yeast mitoplasts and 25°C for rat-liver mitoplasts in a succinate-containing medium.

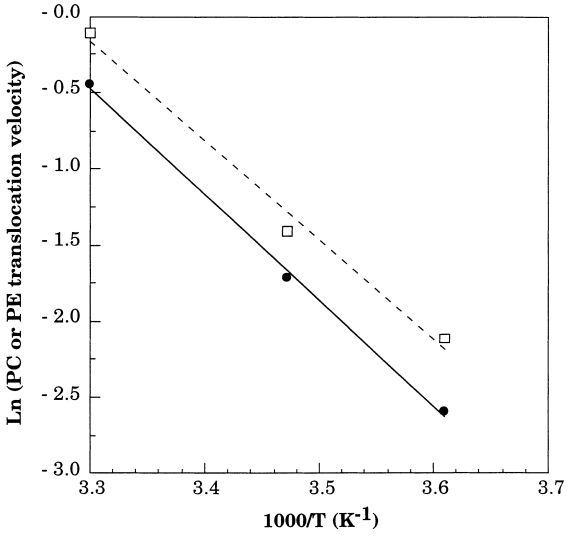


Fig. 2. Translocation rates of PC* and PE* in beef-heart mitochondria at different temperatures. PE* (□) and PC* (●) were incubated in the presence of inner mitochondrial membranes (5 nmol/mg protein) at 4°C, 15°C and 30°C in a medium containing 250 mM sucrose, 2 mM Tris–HCl buffer (pH 7.3) and 20 mM succinate. The phospholipid analogues on the outer leaflet were measured by the BSA extraction method as described in Section 2. Data were expressed as the log of initial velocity vs. the inverse of the incubation temperature.

(NEM) was studied at 30°C under energisation conditions. PC* movement and distribution were insensitive to membrane treatment by NEM. On the other hand, addition of 1 or 5 mM NEM decreased PE* diffusion velocity from 4.20% to 1.85% $min^{-1}$, a value similar to that found in non-energised state (Table 1). The percentage of PE* inside decreased to the level attained with PC*.

### 3.5. *Transmembrane diffusion and equilibrium distribution of cardiolipin*

The spin-labelled cardiolipin cannot be extracted from the membrane by BSA, consequently the transmembrane distribution was determined by addition of ascorbate. A relatively high concentration of ascorbate was necessary since the probe is buried in the hydrophobic part of the membrane. Panel A in Fig. 4 shows that the line shape was representative of a strongly immobilised probe. A typical curve of CL* reduction by ascorbate is shown in Fig. 4B. Two reduction rates are apparent, the faster one corresponds to the reduction of cardiolipin molecules on the outer leaflet of the membrane while the slower one corresponds to the reduction of the spin-labelled phospholipids on the inner leaflet. Very likely, the slope of the slow reduction is associated with the time-scale of ascorbate diffusion through the membrane. An alternative explanation would be that this slope reflects the outward movement of spin labels. Extrapolation of the second slope to time zero on this semi-log scale enables one to obtain the percentage of phospholipid analogue on the inner leaflet at a given time. However, since each cardiolipin distribution measurement involved centrifugation of mitoplasts and reduction by ascorbate during at least 20 min, the first data point was obtained only after an incubation time of 30 min and only a few points were determined. Approximately 40% of the spin-labelled cardiolipin was found on the inner leaflet of the membrane after 30 min incubation at 30°C and 50% after 120 and 150 min incubation. This indicated that the cardiolipin movement was also rapid, the velocity being approximately 2.6% $min^{-1}$ and the half-time 13 min. As a control, more than 90% of CL* was quickly reduced when ascorbate was added to Triton X-100-treated membranes. These experiments suggest that the transmembrane distribution of CL* between the two leaflets was roughly 50/50 at equilibrium. However, a substantial fraction of the

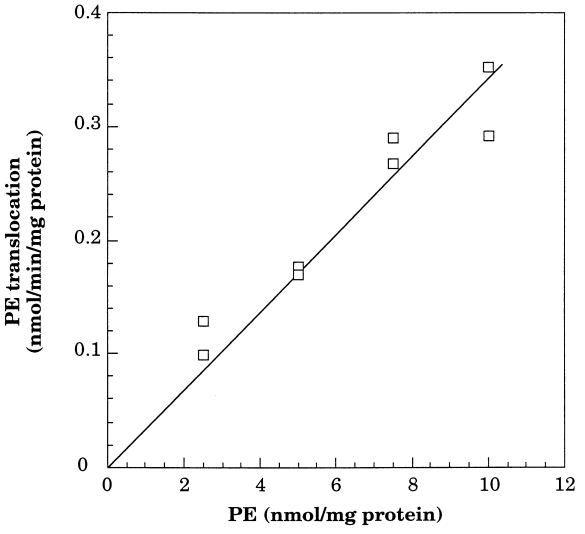


Fig. 3. Reorientation rate of PE* as a function of analogue concentration. Beef-heart mitoplasts were incubated at 30°C in a medium containing 250 mM sucrose, 2 mM Tris–HCl buffer (pH 7.3), 20 mM succinate and various amounts of PE* (from 2.5 to 10 nmol/mg protein). Diffusion kinetics were performed as indicated in Section 2.

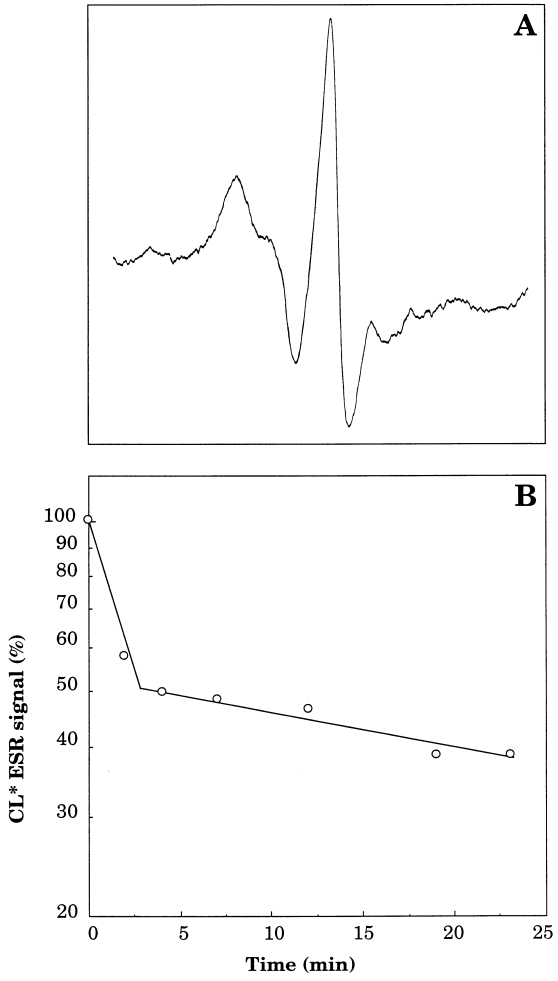


Fig. 4. (A) ESR spectrum of spin-labelled cardiolipin (CL*) incorporated in beef-heart inner mitochondrial membrane. (B) Kinetics of reduction of CL* embedded in inner mitochondrial membranes at 4°C by 100 mM ascorbate. For this experiment mitoplasts (4 mg protein) were incubated with 10 nmol spin-labelled cardiolipin during 150 min at 30°C. The projection of the second slope on the semi-logarithmic scale allows one to estimate the percentage of CL in each leaflet.

spin-labelled cardiolipin is spontaneously reduced (about 10% in half an hour at 4°C), most likely due to the reducing character of the matrix content even in the non-energised state.

## 4. Discussion

We have investigated the transmembrane distribution of the three main phospholipids of the inner mitochondrial membrane by using spin-labelled analogues. In the case of PC* and PE* the nitroxide is at the fourth position of a 5-carbon β chain, while the α chain is a long chain. Such probes, as shown previously with various cell membranes, can be incorporated easily in the outer monolayer without requiring a phospholipid exchange protein, and their transmembrane distribution can be assayed by back exchange using BSA and ferricyanide to reoxidise any reduced probe.

In beef-heart mitochondria, we found that, at equilibrium, approximately 60% of the PE* and 70% of the PC* were located on the outer leaflet of the inner membrane. A similar distribution of PC* and PE* was found in rat-liver as well as in yeast mitoplasts. The uncertainties that we have indicated in Section 3 as well as in Table 1 are means of several experiments carried in the same conditions. As indicated in Section 2, there is a slight contamination by a small fraction of outer membranes that could justify an increase in the uncertainty by a percentage which is difficult to estimate but could be up to 10%. However, as discussed below, the differences in the published data concerning as well the transmembrane distribution and the rate of phospholipid flip-flop in the inner mitochondrial membrane exceeds by far this uncertainty.

When comparing our results to the published ones, different situations occur: the PC distribution that we propose (70% outside) is identical to the one found by Krebs et al. [23] in beef heart but different from the symmetric localisation described in pig heart [38] or rat liver [39]. As for PE, our results with the short-chain spin-label PE* suggest that 60% of the PE is outwardly oriented which is different from Krebs et al. results (40% outside) and much less asymmetric than the 91% outside reported for rat liver mitochondria [39].

How can one explain such discrepancies? The relevance of investigations based on the use of exogenous phospholipase $A_2$ to determine the transmembrane orientation of phospholipids in membranes which are not stabilised by a cytoskeletal network may in fact be questionable. It was shown recently that giant liposomes are very unstable when attacked by phospholipase $A_2$ [40,41]. In addition, mitochondrial membranes contain very little cholesterol, hence flip-flop may be faster than in plasma membranes [42]. Of course, the use of probes is questionable

also. The actual equilibrium distribution of phospholipids depends upon the nature of the acyl chain and not only on the head group [43]. A nitroxide or a fluorescent group can influence the steady-state distribution and the kinetics of redistribution within a membrane [44] and spin labels with one short chain might have slightly different transmembrane distributions than long-chain endogenous phospholipids. However, as revealed from numerous studies in red cells carried out with spin labels, one should anticipate only small variations of flip-flop rates and transmembrane distribution. In a previous publication, we measured the transmembrane distribution and rate of diffusion of long-chain spin-labelled PS and PC in the human red cell membrane and the results obtained were very close to those obtained with spin labels with one short chain and one long chain [45]. Furthermore, the transmembrane diffusion and equilibrium distribution of long-chain radioactive phospholipids were measured by Tilley et al. [46] in the red cell membrane and, although in the latter case the first point can be determined only after 1 h incubation, overall their results matched very well the results obtained with the short-chain spin-labelled lipids in red cells. Hence, the difference in behaviour between short- and long-chain lipids is rather subtle and cannot explain the difference between $t_{1/2} \approx 10$ min found here and $t_{1/2} \approx 24$ h reported in [21].

In this perspective, the results of Rousselet and collaborators [21] on phospholipid flip-flop in bovine inner mitochondrial membranes are surprising. The authors concluded from an investigation with long-chain spin-labelled phosphatidylcholine that the half time of PC flip-flop could be greater than 24 h in the rat-liver inner mitochondrial membrane. Thus, the main issue addressed in the present article is whether the diffusion of phospholipids in inner mitochondrial membrane is very fast (minutes) or very slow (hours) as suggested by Rousselet et al. Here we found that when the mitoplasts were not energised, both PC* and PE* traverse the bilayer with a $t_{1/2}$ of 12 to 15 min. Incubation in a succinate-supplemented medium, in which the mitoplasts were energised, accelerated approximately two times the PE* diffusion rate, without affecting that much that of PC*. Rousselet et al. [21] found after incorporation of long-chain paramagnetic analogues a transmembrane distribution at time $t = 0$, which varied with the method used to label the membrane: incorporation with a PC-exchange protein gave approximately 20% of PC inside while fusion of sonicated liposomes gave 30–35% of PC inside. In the former case (PC exchange protein) the transmembrane distribution did not change significantly after 3 h incubation at room temperature (see Fig. 3a of [21]). Rousselet et al. [21] concluded that PC flip-flop in mitochondrial membranes was a very slow process as it is in erythrocyte plasma membrane. An alternative interpretation could be that the equilibrium was already reached when starting the investigation of the flip-flop rate in Rousselet's experiment because the flip-flop assay had to be preceded by a 30-min incubation at 25°C in the presence of the phospholipid exchange protein. This interpretation allows one to reconcile the 1976 experiments with the present ones. In Rousselet's article, in some experiments the probes were introduced by vesicle fusion. However, in the latter case, because probes are introduced on both leaflets of the mitochondrial membrane, the situation may necessitate a more elaborate analysis.

In summary, we now believe that the rate of phospholipid flip-flop in the mitochondrial membrane is relatively fast with a $t_{1/2}$ of the order of 10–15 min at 30°C and faster at 37°C, thus probably not very different from the very short $t_{1/2}$ found by Button et al. [13] in rat-liver microsomes with the same spin-labelled PC molecule. Dolis et al. [19] also showed that PC equilibrated rapidly over the outer mitochondrial membrane at 30°C ($t_{1/2}$ less than 2 min).

Thus the velocity of the transmembrane reorientation experienced by the different lipids is close to the one reported in membranes in which flippases are involved, such as the non-specific flippase in endoplasmic reticulum membranes [44]. However, when studying the PC* and PE* reorientation in energised mitoplasts as a function of the amount of analogue added to the membrane, the initial velocity remained constant (approximately 4%/min for PE* and 2%/min for PC*). This absence of saturability argues against the involvement of a protein carrier. Addition of NEM to membranes did not modify PC* diffusion properties while affecting slightly PE* distribution which turned to be similar to that of PC*. However, this could be due to an indirect effect of NEM. Therefore, phospholipids may experience a passive diffusion between the two membrane leaflets.

PE* and PC* translocation rates increased with the incubation temperature and the changes were similar for PC* and PE*. This might indicate that the mechanisms of reorientation of these phospholipids are identical.

From studies involving phospholipases $A_2$ [38,39] or antibodies [23], CL is generally described in the literature as being located essentially in the matrix leaflet. By studying the specific binding of dyes to CL, Cheneval et al. [47] and Petit et al. [48] reported a much less asymmetrical distribution (57% outside) which is in the range of the distribution reported here. However, the interpretation of the spin-labelled cardiolipin data in mitochondria has to be made cautiously. Indeed, because of four long-chain fatty acids, the technique of back exchange with reoxidation by ferricyanide was inapplicable. As explained above, the transmembrane distribution was assayed by measuring the signal intensity after ascorbate addition. However, a fraction of the spin-labelled cardiolipin present in the inner leaflet was chemically reduced by the matrix during the incubation, hence reducing the apparent fraction of spin label in the inner leaflet. It is not possible to quantitate this fraction by reoxidation with ferricyanide since labels on both leaflet would be indistinguishable. We can nevertheless make some conclusions from the results obtained with CL*: (i) there is a substantial fraction of spin-labelled cardiolipin which stays after equilibration in the outer monolayer since Fig. 4 indicates clearly that there are two different pools of CL* present, one being easy to reduce by ascorbate added externally and a second pool more difficult to reach; (ii) the apparent equilibrium in the transmembrane distribution of CL* is reached in about 90 min at 30°C. Thus, CL* can flip from the outer to the inner monolayer in a relatively short period of time. This result is consistent with our previous study [49] which showed that neosynthesised CL is rapidly (1 h) redistributed between the two leaflets of the inner mitochondrial membrane of *Saccharomyces cerevisiae* during the switch from fermentative to gluconeogenic growth.

## 5. Conclusions

In the present article we show that the transverse diffusion of PC, PE and even CL spin labels in mitoplasts is rapid, in agreement with most other measurements of lipid flip-flop in organelle membranes. This may be a general feature of inner membranes of eukaryotic cells, possibly associated with their low levels of cholesterol in contrast with that of plasma membranes. The absence of cholesterol cannot explain by itself the rapid diffusion since much slower diffusion rates have been reported in liposome containing no cholesterol, thus the presence of the proteins is certainly an important factor. The particularly high protein to lipid ratio in mitochondria is likely to create many local defects, or mismatches, between the lipid bilayer and the protein interface. However, we found no conclusive evidence of a specific transporter or 'flippase'. The involvement of non-bilayer structures in mitochondria cannot be ruled out; however, we have no elements here to support this hypothesis put forward by Cullis et al. [20] and that could explain the rapid passage of lipids. The stimulation of PE* translocation in the energised state compared to that obtained in the non-energised state or when membranes are NEM-treated is less than a factor of 3; this small stimulation does not suggest the existence of a specific enzyme. The higher initial rate of PE* in the energised state could be an indirect effect on lipid translocation, for example, associated with the change in transmembrane potential and/or the reorganisation of proteins in the energised state.

If the phospholipid passage is catalysed in a non-specific manner, in other words if lipid flip-flop is only a real diffusion process, then in order to explain the asymmetrical distribution of the probes at the steady state, which appears to be lipid-specific, one must implicate: (i) specific lipid–protein interactions and (ii) an asymmetrical distribution of the mitochondrial proteins.

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