Biochimica et Biophysica Acta, 426 (1976) 372–384

BBA 77223

STUDY OF THE TRANSVERSE DIFFUSION OF SPIN-LABELED PHOSPHOLIPIDS IN BIOLOGICAL MEMBRANES

II. INNER MITOCHONDRIAL MEMBRANE OF RAT LIVER: USE OF PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN

ANNIE ROUSSELET*, ANNETTE COLBEAU**, PAULETTE M. VIGNAIS** and PHILIPPE F. DEVAUX*

**Biophysique Moléculaire, GPS-ENS, Tour 23, Université Paris VII - 2 place Jussieu 75221 Paris Cedex 05 and **DRF/Biochimie, CEN-G., B.P. 85 38041 Grenoble-Cedex (France)*

(Received July 30th, 1975)

SUMMARY

Spin-labeled phosphatidylcholine was incorporated into the membrane of isolated "inner membrane+matrix" particles of rat liver mitochondria by incubation with sonicated spin-labeled phosphatidylcholine vesicles at 22 °C. When the spin label was on the acyl chain the incorporation of phosphatidylcholine into the membrane was stimulated by the presence of the phosphatidylcholine exchange protein extracted from rat or beef liver. On the other hand no stimulation was observed when the nitroxide was on the polar head-group.

When spin-labeled phosphatidylcholine was incorporated into the mitochondrial membrane in the absence of phosphatidylcholine exchange protein, ascorbate treatment at 0 °C reduced the EPR signal of the spin-labeled membranes by approximately 50 %, indicating that fusion incorporates molecules equally on both sides of the membrane. On the other hand when spin-labeled phosphatidylcholine was incorporated in the presence of the exchange protein most of the EPR signal could be destroyed by the ascorbate treatment at 0 °C, indicating that the spin-labeled phosphatidylcholine had been selectively incorporated in the outer layer of the membrane. Finally when the label is on the polar head-group the inner content of mitochondria reduces the label facing the matrix, thus creating again an anisotropy of the labeling.

The anisotropic distribution of spin-labeled phosphatidylcholine in the mitochondrial membrane was found to be stable at 25 °C for more than 2 h. It is therefore concluded that the rate of outside-inside and inside-outside transitions are extremely slow (half-life greater than 24 h).

INTRODUCTION

The study of phospholipid transverse diffusion in biological membranes with spin-labeled analogs of phosphatidylcholine involves the incorporation of artificial-

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

lipids into the membrane. In our previous study on red blood cells [1] fusion of sonicated artificial vesicles was used to incorporate the spin labels in the erythrocyte membrane. The fusion process results in the net addition of phospholipids. A more natural process to incorporate phospholipids involves the use of a phospholipid exchange protein. Exchange of phosphatidylcholine between artificial vesicles and biological membranes can indeed be induced by a soluble exchange protein isolated from liver cells [2]. This enzyme is not functional in the presence of red blood cells (Wirtz, K., personal communication) but stimulates the incorporation of spin-labeled phosphatidylcholine in mitochondrial membranes.

In this paper we will present results on the transverse diffusion of spin-labeled phospholipids in rat liver inner mitochondrial particles, using the phosphatidylcholine exchange protein described by Kamp et al. [10]. Our results will also give some insight on the problem of incorporation of phospholipids in this membrane.

MATERIALS AND METHODS

Chemicals

Fatty acid-free bovine serum albumin was purchased from Pentex (Miles Laboratoires, USA). Egg lecithin and phosphatidic acid, derived from egg lecithin treated by phospholipase D, were gifts from Dr. Marguerite Faure (Institut Pasteur, Paris).

Spin-labeled molecules used in this work are described elsewhere [1]. Radioactive [^{32}P]phosphatidylcholine was isolated from the livers of rats which had received an intraperitoneal injection of [^{32}P]phosphate 8 h prior to killing.

Membrane preparation

Rat liver mitochondria were purified from a 10 % homogenate in 0.27 M sucrose/2 mM Tris · HCl (pH 7.4) by conventional differential centrifugation and washed three times with the same medium. Inner membranes plus matrix particles (low speed pellet) were prepared by treatment with 20 mM phosphate buffer (pH 7.4) according to Parsons and Williams [3]. These mitochondrial particles were then resuspended in isotonic sucrose solution. Cytochrome oxidase was determined according to Appelmans et al. [4] and malate dehydrogenase according to England and Siegel [5]. These proteins were used as marker enzymes. The protein concentration was measured by the biuret method.

Lipid extraction and analysis

Membrane lipids were extracted according to Dawson et al. [6] and lipid phosphorus was determined after wet ashing by the method of Barlett [7]. Lipid peroxides were assessed by the amount of malonaldehyde formed with thiobarbituric acid [8]. Ascorbic acid was measured by the reduction of 2,6-dichloroindophenol [9].

Isolation of phosphatidylcholine exchange protein

A crude preparation of the phosphatidylcholine exchange protein was obtained by submitting the cytosol proteins of rat liver to partial denaturation at pH 5.1 for 2 h according to Kamp et al. [10]. The cytosol fraction of a 10 % liver homogenate in 0.27 M sucrose/2 mM Tris · HCl (pH 7.4) is the supernatant fluid obtained after 1 h centrifugation at 100 000 $\times g$. This high speed centrifugation allows the removal of

organelles, in particular the lysosomes which would otherwise release soluble proteolytic and lypolytic enzymes during the acid treatment. After removing the precipitated proteins by centrifugation (15 000 × *g*, 15 min), the pH of the clear solution was brought back to 7.4 by addition of Tris base. The protein concentration of the solution varied between 5 and 9 mg protein/ml. This crude extract of phosphatidylcholine exchange protein was used in some experiments described below. When the ratio of incorporated labeled phosphatidylcholine to total membrane phospholipids was determined, values as high as those obtained with the pure enzyme were obtained. The pure phosphatidylcholine exchange protein used in this work was a gift from Dr. K. W. A. Wirtz (Utrecht).

Incorporation of labeled phosphatidylcholine into mitochondrial membranes

(*a*) *Liposome preparation.* Suspensions of spin-labeled phosphatidylcholine in buffer (0.27 M sucrose/10 mM Tris · HCl, pH 7.8) were sonicated for approximately 5 min at 22 °C, using a Branson sonifier at about half of the maximum output. After sonication, the suspensions were centrifuged for 20 min in an Eppendorf minicentrifuge to eliminate the larger liposomes and titanium particles. The phosphatidylcholine concentration of the emulsion was about 1 μmol lipid P/ml and contained 2–4 % of phosphatidic acid. 2,2,6,6-Tetramethylpiperidine-*N*-oxyl-labeled phosphatidylcholine (TEMPO-phosphatidylcholine) had to be diluted with egg lecithin (molar ratio 1 to 1) to obtain a clear solution.

(*b*) *Incorporation of labeled phosphatidylcholine molecules into mitochondrial particles by fusion.* Labeled phosphatidylcholine molecules emulsified as described above (a) were mixed with inner mitochondrial membrane particles. After incubation at room temperature the membrane particles were centrifuged (15 000 × *g*, 10 min) and washed twice with buffered isotonic sucrose containing 2 % fatty acid-free bovine serum albumin. In a typical experiment, 0.2 μmol of a phosphatidylcholine suspension in about 0.4 ml was mixed with inner membrane particles (6 mg of proteins, final volume 1 ml). After 1 h incubation at 22 °C, the amount of (10, 3) phosphatidylcholine incorporated represented about 2 % of the total phospholipids. It was about ten times lower with TEMPO-phosphatidylcholine.

(*c*) *Incorporation of labeled phosphatidylcholine molecules in mitochondrial particles in the presence of phosphatidylcholine exchange protein.* Typical incubation conditions were as follow. 0.35 μmol of sonicated labeled phosphatidylcholine (0.4 ml) were added to 4 ml of crude phosphatidylcholine exchange protein (7 mg protein/ml) or to 4 ml of buffer (0.24 M sucrose 20 mM Tris · HCl, pH 7.8) with 20 μl of purified exchange protein containing 10 units in 40 % glycerol; one unit of activity is expressed as the transfer of 1 nmol P/min. The mixture was incubated for 30 min at 25 °C to charge the exchange protein with labeled phosphatidylcholine. Inner membrane+matrix particles (6 mg protein) were then added together with fatty acid-free bovine serum albumin (2 % final concentration) and EDTA (1 mM final concentration). The incubation was allowed to proceed for 30–90 min at 20 °C then ended by cooling the tubes in an ice bucket and centrifuging at 4 °C in a Sorvall centrifuge at 15 000 × *g* for 10 min. The pellet was washed twice with 2 ml of 0.27 M sucrose/10 mM Tris · HCl (pH 7.8)/1 % bovine serum albumin, using an Eppendorf

minicentrifuge. The pellet was suspended in 50 μl of the surcrose/Tris solution.

A control tube, where the exchange protein was replaced by sucrose solution, allows us to estimate the amount of labeled phospholipid incorporated by fusion of the phosphatidylcholine vesicles with the mitochondrial membrane.

Measurements of the spin-labeled phosphatidylcholine incorporation

The molar ratio of spin-labeled phosphatidylcholine incorporated into the mitochondrial membrane was determined by first extracting the lipids. The intensity of the EPR signal of a known amount of extracted phospholipids dissolved in chloroform was compared to a reference solution of spin-labeled phospholipids in chloroform.

Measurement of the reduction of nitroxide radicals by ascorbate

To measure the reduction of the nitroxide radicals incorporated into inner mitochondrial membranes, 100 μl of a 10 or 20 mM sodium ascorbate solution (in 0.27 M sucrose/10 mM Tris · HCl, pH 7.4, 0 °C, freshly prepared) was added to 100 μl of a membrane suspension. After rapid mixing, an aliquot was used to record the EPR spectra at 0 °C in a 50 μl glass capillary with a Thompson TSN-254-2 EPR spectrometer provided with a temperature control accessory. Different aliquots were used to obtain the evolution of the signal intensity of a given sample when incubated with ascorbate at 0 °C. The reduction by ascorbate was followed and monitored as previously described [1].

RESULTS

(1) Incorporation of spin-labeled analogs of phosphatidylcholine into inner mitochondrial membrane particles can be stimulated by the presence of phosphatidylcholine exchange protein

The activity of the exchange protein was tested using spin-labeled and radioactively labeled phosphatidylcholine. Figs. 1 and 2 show that (10, 3)phosphatidyl-

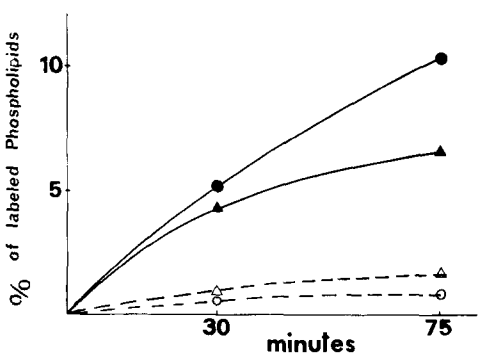

Fig. 1. Comparison of the incorporation of [^{32}P]phosphatidylcholine (●, ○) and (10,3)phosphatidylcholine (▲, △) into inner mitochondrial particles, at 22 °C: (—) in the presence of phosphatidylcholine exchange protein (5.4 mg/ml) (- - -); without phosphatidylcholine exchange protein. Incubation medium is described in Material and Methods. Crude protein preparation was used for this experiment.

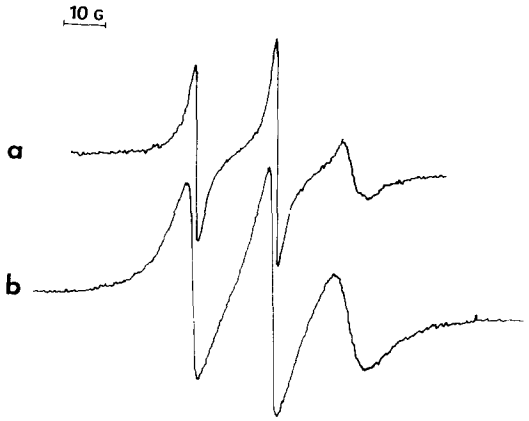

Fig. 2. EPR spectra of (1,14)phosphatidylcholine incorporated into inner mitochondrial particles: a, without phosphatidylcholine exchange protein; b, in the presence of phosphatidylcholine exchange protein. Incubation medium containing crude preparation of phosphatidylcholine exchange protein was used in b and replaced by buffer in a. Incubation time: 90 min at 22 °C. Since the same gain was used for the recording of both spectra the stimulation appears only in the very marked broadening of the lines. Approximate amounts of labeled phospholipids incorporated: a, 2 % (molar ratio); b, 10 % (molar ratio).

choline and (1, 14)phosphatidylcholine can be taken up by the exchange protein and catalytically incorporated into isolated inner mitochondrial membranes. However, the efficiency of catalysis when a spin-labeled molecule is used as substrate is lower than with [^{32}P]phosphatidylcholine. The incorporation levels off much more rapidly with (10, 3)phosphatidylcholine than with [^{32}P]phosphatidylcholine. A preferential incorporation of [^{32}P]phosphatidylcholine is clearly illustrated when mixed liposomes are used. If a 1 : 1 mixture of [^{32}P]phosphatidylcholine and (10, 3)phosphatidylcholine vesicles are incubated with inner mitochondrial particles, after 75 min a 2-fold excess of [^{32}P]phosphatidylcholine over (10, 3)phosphatidylcholine is found in the mitochondrial membranes. However, in the control experiment performed without phosphatidylcholine exchange protein an equal incorporation of both labeled phosphatidylcholine molecules was obtained.

Experiments performed at different temperatures show that the rate of exchange increases with temperature as does the amount of labeled phosphatidylcholine incorporated by fusion. For example, the ratio of phosphatidylcholine incorporated by exchange to phosphatidylcholine incorporated by fusion is roughly the same at 22 and at 0 °C. A temperature of incubation higher than 22 °C was not used, to preserve as much as possible the integrity of the membrane structure. For the same reason membranes were never incubated or studied over periods exceeding 3–4 h at room temperature. Indeed inner mitochondrial membranes contain endogenous phospholipase A [11] capable of altering the membrane integrity after too long periods at or above room temperature. Incubation periods in the range of 1 h at 22 °C were nevertheless necessary to obtain enough incorporation of spin-labeled phosphatidylcholine in mitochondrial particles for further studies. (Under such conditions 2–4 % of the phospholipids were labeled in the membrane.)

TEMPO-phosphatidylcholine, a phosphatidylcholine labeled on the polar

head-group can also be incorporated in inner mitochondrial particles when mixed sonicated vesicles of TEMPO-phosphatidylcholine and egg lecithin are incubated with the mitochondrial membranes. However, no stimulation of the incorporation of TEMPO-phosphatidylcholine was detected in the presence of phosphatidylcholine exchange protein. This could be due to the nitroxide on the polar head-group, or alternatively to the presence of saturated acyl chains.

(2) Incorporation of spin-labeled phosphatidylcholine by fusion creates a symmetrical distribution of labeled phospholipids between both sides of the membrane

Sodium ascorbate was used to determine the sideness of the distribution of spin-labeled phospholipids incorporated by fusion in inner mitochondrial membranes. We found that high concentrations of ascorbate (10–20 mM) as well as long periods of incubation (0.5–3 h) at 0 °C had to be used to reduce totally the label theoretically accessible to ascorbate.

Such conditions could eventually result in a progressive penetration of ascorbate into the matrix of mitochondria or, alternatively, in a deterioration of the particles. The measure of the reduction of 2,6-dichloroindophenol did not allow us to detect any amounts of reduced ascorbate inside the particles. We were also unable to detect any change in the membrane integrity by measuring the cytochrome oxidase activity or the amount of membrane peroxides, the same values being obtained in the control samples and in ascorbate-treated particles. However, it has been possible to show (Table I) that ascorbate treatment made the membrane more leaky. When malate dehydrogenase, a marker enzyme of the matrix space, was measured in the supernatant fluid obtained by resedimenting the particles after ascorbate treatment at 0 °C, it was found that more matrix proteins were released from the samples that had been in contact with ascorbate (Table I).

TABLE I

PROLONGED INCUBATION AT ROOM TEMPERATURE AND WITH HIGH CONCENTRATION OF ASCORBATE AT 0 °C INCREASE THE RELEASE OF MATRIX PROTEINS FROM THE MITOCHONDRIAL PARTICLES

33 mg of inner membrane + matrix particles in isotonic sucrose (1.4 ml final volume) were incubated for 2 h at 0 °C with or without 20 mM ascorbate at pH 7.4. At the end of the incubation period 3 ml of isotonic sucrose were added and the particles removed by centrifugation. The amount of proteins and the malate dehydrogenase activity were measured in the supernatant fluid. Cytochrome oxidase activity and the lipid peroxides were determined in the pellet. No significant differences were found for the latter between the various samples.

	Ascorbate (20 mM 0 °C)	Proteins released		Malate dehydrogenase released (total activity)
		mg	%	
No preincubation	–	1.5	4.5	1.9
at 22 °C	2 h	4.7	13.5	3.2
Preincubation in	–	3.6	11	6.1
isotonic sucrose for 2 h at 22 °C prior ascorbate treatment	2 h	7.6	23	11.1

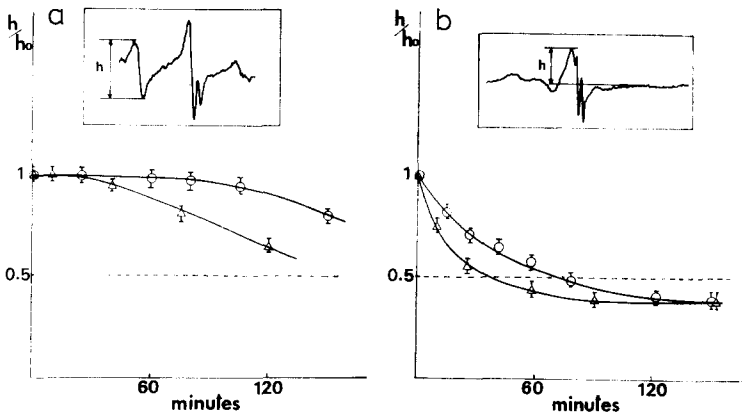

Fig. 3. Rate of reduction of (a) (1,14)phosphatidylcholine and (b) (10,3)phosphatidylcholine incorporated by fusion in inner mitochondrial particles. Incorporation is described in the text (Materials and Methods). The incubation time was 1 h and temperature 22 °C. Final sodium ascorbate concentration used for these reductions are (○) 10 mM and (△) 20 mM. In the insets are shown typical EPR spectra of the labeled samples when ascorbate is added. The sharp doublet appearing in the middle has already been reported by several authors to accompany ascorbate oxidation. h is the peak height of the mid field line, h_0 the corresponding height at time $t = 0$.

A different control of the nonpenetration of ascorbate is provided by the comparison of the reduction rates of (10, 3)phosphatidylcholine and (1, 14)phosphatidylcholine incorporated by fusion in inner mitochondrial particles. In (10, 3) phosphatidylcholine, the nitroxide is placed near the polar head-group, whereas in (1, 14)phosphatidylcholine it is near the methyl terminal of the β chain, hence in the middle of the bilayer when (1, 14)phosphatidylcholine is intercalated in a membrane. One should expect, therefore, (1, 14)phosphatidylcholine to be completely protected from ascorbate reduction whereas the fraction of the (10, 3)phosphatidylcholine molecules, outwardly exposed, should be available to ascorbate reduction. The difference in accessibility of the two spin labels should at least affect the rate of their reduction by ascorbate. This has already been shown in the erythrocyte with (10, 3)phosphatidylcholine and (7, 6)phosphatidylcholine [1].

Fig. 3 shows the very striking difference in the kinetics of reduction of the signal coming from the two labels. With (10, 3)phosphatidylcholine, the amplitude of the EPR signal in the presence of 20 mM ascorbate at 0 °C decreases in about 60 min to a plateau slightly below 50 % of the initial amplitude. On the other hand, with (1, 14)phosphatidylcholine the signal stays constant for about 1 h then gradually decreases.

In summary, at 10–20 mM concentrations and at 0 °C, ascorbate afforded a selective reduction of the outwardly exposed spin labels. The fact that, with (10, 3) phosphatidylcholine, the intensity of the ESR spectra reaches approximately 50 % of its original value after ascorbate treatment, indicates that only half of the incorporated (10, 3)phosphatidylcholine molecules are accessible to ascorbate. This means that the spin labels are equally distributed on both sides of the membrane. As indicated in Fig. 3 the time constants for the reduction of the two spin labels used depend very much on the ascorbate concentration. With ascorbate concentrations below 5 mM the reduction was too slow to be conveniently measured.

Experiments with different levels of incorporation of the spin-labeled lipids gave the same characteristic figures, however the molar ratio of spin-labeled to unlabeled phospholipids was kept below approx. 5 % with (10, 3)phosphatidylcholine and approx. 2 % with (1, 14)phosphatidylcholine. These concentrations correspond to those where spin-spin interactions appear. At higher levels, the amplitude of the EPR signal is no longer proportional to the spin concentration (see Fig. 2).

(*3*) *In the presence of phosphatidylcholine exchange protein a selective labeling of the outer layer is created*

In the experiments described in Fig. 4 the mitochondrial particles incubated with (10, 3)phosphatidylcholine in the presence of exchange protein were centrifuged, resuspended in buffered isotonic sucrose and then immediately exposed to ascorbate (10 mM) at 0 °C. The EPR signal decreased very significantly below 50 % of the original value. The level at which a plateau, or at least a "quasi-plateau", is reached in this type of experiment depends upon the efficiency of the stimulation of the spin-labeled phospholipid incorporation by the exchange protein. The larger the amount of spin label incorporated by exchange, the lower the plateau. Different ascorbate concentrations led essentially to a change in the time scale of the reduction process but to no significant differences in the maximum degree of reduction. From these experiments it is concluded that the exchange protein creates a preferential labeling of the outer layer. It may be of interest to mention that practically the same degree of incorporation of labeled phosphatidylcholine was obtained whether a crude preparation or a highly purified exchange protein was used. The same slopes in the reduction curves were also observed, indicating that contaminants in the crude preparation did not interfere with the phospholipid exchange in the membrane or its distribution in the membrane.

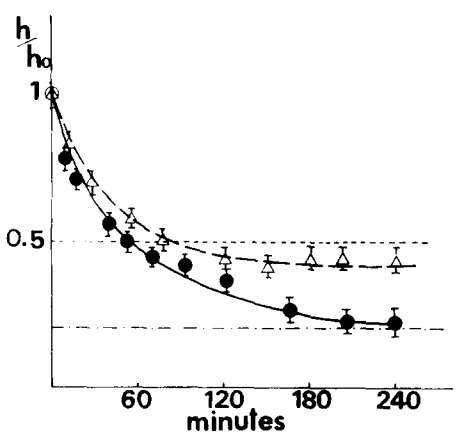

Fig. 4. Kinetics of the reduction of (10,3)phosphatidylcholine incorporated with (●) or without (△) phosphatidylcholine exchange protein. Highly purified phosphatidylcholine exchange protein was used, incubation time was 1 h and temperature 22 °C. Incubation conditions as described in Materials and Methods. The lower dashed line indicates the expected ratio between inside and outside labeling as determined by the control experiment and assuming that fusion creates equal labeling on both sides and exchange only on the outside.

(4) Study of the randomisation of an anisotropic labeling in mitochondrial particles

(a) After incorporation by fusion. The anisotropy of labeling of particles which have been fused with spin-labeled phosphatidylcholine vesicles is obtained by incubation of the particles with 10 mM ascorbate for approximately 2 h (or 20 mM for 1 h) at 0 °C. The mitochondrial particles are then centrifuged and washed to remove ascorbate from the suspending medium. They are resuspended in 0.27 M sucrose and incubated at room temperature for 0, 1, 2 or 3 h before being submitted to a second ascorbate treatment. Typical results showing the observed reduction rates are given in Fig. 5. It appears that the initial slopes of the second reduction experiments depend on the incubation period. The reduction curves do not seem to level off very clearly during the second ascorbate treatment. However, from the modification of the slope it can be inferred that a very significant change in the spin label distribution takes place during incubation.

(b) Use of phosphatidylcholine exchange protein. As shown in the previous sections, the loading of the membranes with (10, 3) phosphatidylcholine in the presence of the exchange protein creates a preferential labeling of the outer layer of the membrane. Even if the stimulation is small, the outside/inside ratio of labeled phosphatidylcholine will be large. In other words, a small stimulation creates a large anisotropy.

To measure the evolution of this anisotropic distribution of phospholipids, different samples of the same mitochondrial membrane preparation which had been labeled by incubation in the presence of the exchange protein were maintained at 22 °C for different periods of time, then chilled to 0 °C and treated with ascorbate. Typical results are shown in Fig. 6a. After 3 h at 22 °C, only very little modification

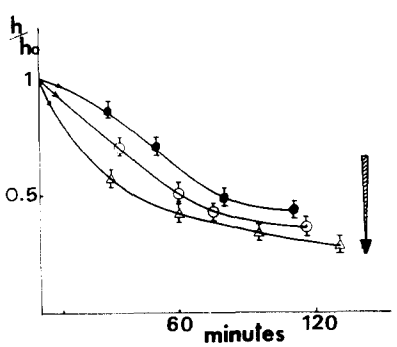

Fig. 5. Study of the flip-flop of phospholipids in the inner mitochondrial membrane of rat liver by the kinetics of reduction of (10,3)phosphatidylcholine incorporated by fusion. After spin-labeled phospholipids had been incorporated by fusion into mitochondrial particles, 20 mM sodium ascorbate was added at 0 °C for 1 h to remove the labeling of the outer layer. Then ascorbate was removed by centrifugation and the membranes are resuspended in Tris buffered sucrose 0.27 M and at 22 °C for various periods of time. A second ascorbate treatment was then undertaken (20 mM at 0 °C) and the results of these treatments are shown in the figure. The time of incubation at room temperature was (●) 30 min; (○) 2 h and (△) 3 h. Modifications of reduction curves (indicated schematically by the arrows) show that after a long incubation more and more spin labels are immediately available to the ascorbate. The amplitude of the initial signal was normalised for each sample. However the actual differences in amplitude were very small at the beginning of each ascorbate treatment, indicating that no spontaneous reduction had taken place during incubation at 22 °C.

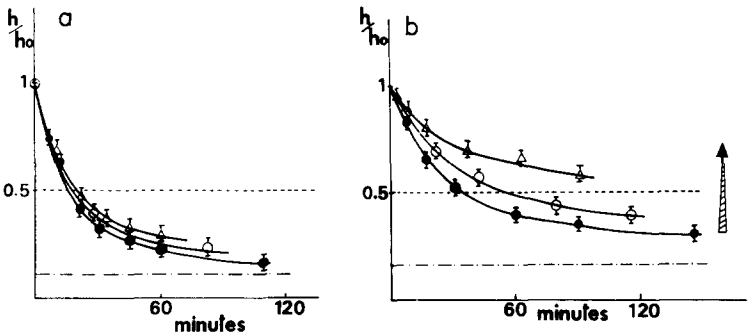

Fig. 6. Study of the flip-flop of phospholipids in inner mitochondrial membranes of rat liver by the kinetics of reduction of (10,3)phosphatidylcholine incorporated in the presence of phosphatidylcholine exchange protein. (a) Membranes are loaded with (10,3)phosphatidylcholine in the presence of crude extract of phosphatidylcholine exchange protein (1 h at 22 °C, incubation medium described in Materials and Methods). After incubation, the membranes are centrifuged and resuspended in Tris · HCl buffer (pH 7.4) sucrose 0.27 M and allowed to stand at room temperature. Sodium ascorbate was added and the spectrum amplitude monitored at 0 °C after various intervals of time. (●) zero; (○) 2 h; (△) 3 h. Very little, if any, modification is seen in the reduction rates. (b) The same procedure is applied except that prior to any operations with spin labels the membranes were incubated for 1 h at 0 °C with sodium ascorbate, then centrifuged to remove ascorbate and resuspended. This time, a clear evolution of the kinetics of reduction appears (as indicated by the arrow). This evolution is consistent with a randomisation of the lipid distribution, taking place in 3–4 h at 22 °C.

is seen. There is almost no increase of the plateau level nor any systematic change in the initial slope of the reduction curves. Similar experiments were done at 35 °C without striking differences in the reduction curves. If the temperature is raised above 35 °C spontaneous reduction of the label occurs.

In conclusion, when (10, 3) phosphatidylcholine is incorporated in the presence of phosphatidylcholine exchange protein, the anisotropic distribution obtained is very stable. On the other hand, when the anisotropy of the labeling is created by ascorbate, substantial redistribution corresponding to a flip-flop of the spin-labeled phospholipids takes place in 2–3 h.

(*c*) *Membranes pretreated with ascorbate.* In an attempt to understand the apparent discrepancy shown in the two preceding experiments, in a new series of experiments the membranes were pretreated with 20 mM sodium ascorbate at 0 °C for 1 h before incorporating the spin labels with the exchange protein. The experiments were then carried out in the same way as described in paragraph (b). Fig. 6b shows that at least the initial slope of the reduction curve depends on the length of incubation at room temperature, indicating that less and less spin-labeled phospholipids become available to ascorbate reduction. This result indicates clearly that in such conditions a flip-flop is detectable.

(*5*) *Further study of the transverse diffusion of phospholipids in the inner mitochondrial membrane using an analog of phosphatidylcholine labeled on the polar head-group*

A saturated phosphatidylcholine molecule containing a 6-membered ring nitroxide on the choline group can be incorporated into inner mitochondrial particles by fusion. When mitochondrial particles containing this phospholipid were treated

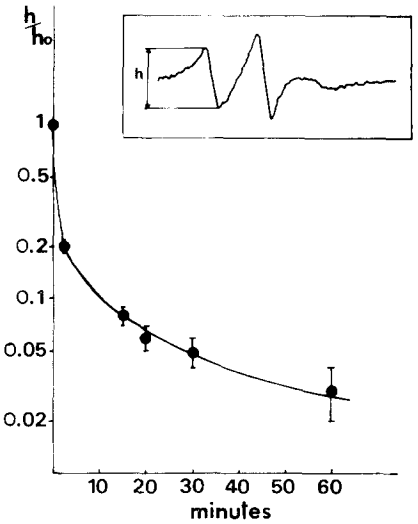

Fig. 7. Reduction at 0 °C by 10 mM ascorbate of TEMPO-phosphatidylcholine incorporated by fusion in inner mitochondrial particles. Note the logarithmic scale of the ordinate. The reduction over a long period does not follow a simple exponential decay. More than 80 % of the signal is destroyed in the first 2 min, indicating that most of the spin-labeled lipids are facing the outer surface. The inset shows the EPR spectrum (in absence of ascorbate) of TEMPO-phosphatidylcholine incorporated in the membrane. Actual percentage of labeled phospholipids was not measured but from the signal amplitude could be estimated as being lower than 1 %.

with ascorbate at 0 °C, the total EPR signal decreased rapidly to zero, indicating that the signal was coming from the outer layer only (Fig. 7). However, molecules incorporated by fusion are found in both the outer and the inner monolayer. Thus the asymmetrical distribution must be the consequence of a selective reduction of the inwardly exposed TEMPO-phosphatidylcholine molecules. Probably glutathione still present in the matrix compartment is responsible for this reduction. The same property was found with red blood cells [1].

We next studied the evolution of the signal arising from the outer layer only. In all our experiments no significant evolution of the signal amplitude could be detected within 2–3 h. Usually after 3 h at room temperature (and earlier at higher temperature) the signal suddenly decreases to zero, probably indicating the lysis of the particles.

DISCUSSION

Our results are concerned with two aspects of membrane formation or turnover, the incorporation of phospholipids and their transverse diffusion in the membrane.

We have verified that a fusion process, as it does in other membranes, incorporates new phospholipids equally well on both sides of inner mitochondrial membranes. Our experiments with inner mitochondrial particles could, however, suggest a slightly asymmetrical incorporation, since we repeatedly obtained the reduction of 55–60 % of the incorporated labels, instead of 50 % as obtained with erythrocytes [1].

But it is probably due to the presence of a small fraction of membrane fragments which are accessible to ascorbate from both sides of the membranes after the various treatments (Table I). Alternatively it is conceivable that fusion creates a small anisotropy in the phospholipid distribution, reflecting the known asymmetry of sonicated vesicles [12].

Our results on the incorporation of spin-labeled phospholipids with the phosphatidylcholine exchange protein provide direct evidence for a selective incorporation in the external monolayer. Indeed when (10, 3) phosphatidylcholine incorporation was activated by the phosphatidylcholine exchange protein (Fig. 4), most of the EPR signal could be removed by ascorbate treatment. Even in the presence of the exchange protein, some fusion of the spin-labeled liposomes with the mitochondrial particles does occur and thus introduces spin labels in the inner monolayer (cf. Fig. 1). It is possible to calculate the theoretical ratio of the inside/outside labeling, and to deduce what the theoretical plateau should be after reduction of the outwardly exposed labels. Such theoretical levels are indicated in Fig. 4 and Fig. 6 and are compared with the actual levels obtained experimentally by ascorbate reduction. Agreement is satisfactory. The finding that phosphatidylcholine exchange protein incorporated on only one side of the membrane can be considered as complementary to the results of Johnson et al. [12]. These authors have shown that the phosphatidylcholine exchange protein only removes phospholipids from the outer layer of artificial vesicles. So in conclusion this soluble protein will interact with membrane surfaces without perturbing any existing asymmetrical distribution of phospholipids.

Finally, by studying the randomisation of an asymmetrical distribution of spin-labeled phosphatidylcholine we have shown that the transverse diffusion must be very slow. Using the exchange protein to achieve an asymmetrical distribution of (10, 3)phosphatidylcholine, no significant modifications in the labeling appeared in 3 h at 22 or 35 °C.

As a change of 10% in signal amplitude can be considered within our experimental error, we conclude that the maximal number of spin-labeled lipids diffusing from one monolayer to the other in 2–3 h at 22 °C is about 10% of the total spin labels. Consequently it can be extrapolated that, at 22 °C, a minimum half-time for flip-flop is 20–30 h.

We have shown that when membranes are incubated in the presence of 10–20 mM ascorbate at 0 °C before any diffusion can take place, the flip-flop rate is substantially accelerated. Characteristic times of the inside-outside as well as the outside-inside flipping of (10, 3)phosphatidylcholine can be estimated from our experiments to 3–5 h at 22 °C. This estimate is based essentially on the fact that a large redistribution of spin-labeled lipids takes place in 3 h, as shown at least by the change in the initial slope of the reduction curves (see Figs. 5 and 6B). Obviously the technique which involves a severe pretreatment of the membrane before measurement of transverse diffusion (described in Section 4) is less reliable than the technique whereby an asymmetric labeling is created without perturbing the membrane. Therefore, the main conclusion that may be drawn from this study is that the flip-flop of phospholipid molecules in mitochondrial membranes is a very slow process. This result is in good agreement with the measurement of the phospholipid flip-flop we have done with human red blood cells [1] and the results of other authors in various model systems [12–14].

Hence flip-flop is not only a negligible event in plasma membranes containing cholesterol, but also in more fluid biological membranes such as the inner mitochondrial membrane of rat liver.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. K. Wirtz for the gift of the purified phosphatidylcholine exchange protein. Dr. A. Bienvenue, C. Guthman and J. Matricon are acknowledged for the gift of spin-labeled phospholipids. Expert technical assistance of Mrs J. Chabert is acknowledged. This work was supported by research grants from the "Délégation Générale à la Recherche Scientifique et Technique" and from the "Centre National de la Recherche Scientifique". Annie Rousselet is a recipient of a fellowship from the D.G.R.S.T.

ADDENDUM

Recent experiments by J. E. Rothman and E. A. Dawidowicz [15] confirm that the flip-flop of phosphatidylcholine in artificial liposomes is very slow.

REFERENCES

1 Rousselet, A., Guthmann, C., Matricon, J., Bienvenue, A. and Devaux, P. F. (1976) Biochim. Biophys. Acta 426, 357–371
2 Wirtz, K. W. A. (1974) Biochim. Biophys. Acta 344, 95–117
3 Parsons, D. F. and Williams, B. R. (1967) Methods Enzymol. 10, 443–448
4 Appelmans, F., Wattiaux, R. and de Duve, C. (1955) Biochem. J. 59, 438–445
5 Englard, S. and Siegel, L. (1969) Methods Enzymol. 13, 99–106
6 Dawson, R. M. C., Hemington, N. and Lindsay, D. B. (1960) Biochem. J. 77, 226–230
7 Bartlett, G. R. (1958) J. Biol. Chem. 234, 466–468
8 Ernster, L. and Norden Brand, K. (1967) Methods Enzymol. 10, 574–580
9 Roe, J. H. (1954) Methods Biochem. Anal. 1, 115–139
10 Kamp, H. H., Wirtz, K. W. A. and Van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 318, 313–325
11 Colbeau, A., Nachbaur, J. and Vignais, P. M. (1971) Biochim. Biophys. Acta 249, 462–492
12 Johnson, L. W., Hughes, M. E. and Zilversmit, D. B. (1975) Biochim. Biophys. Acta 375, 176–185
13 Kornberg, R. D. and McConnell, H. M. (1971) Biochemistry 10, 1111–1120
14 Sherwood, D. and Montal, M. (1975) Biophys. J. 15, 417–434
15 Rothman, J. E. and Dawidowicz, E. A. (1975) Biochemistry 14, 2809–2816