Rapid Kinetics of Insertion and Accessibility of Spin-Labeled Phospholipid Analogs in Lipid Membranes: A Stopped-Flow Electron Paramagnetic Resonance Approach

Uwe Marx,* Günter Lassmann,# Kandatege Wimalasena,§ Peter Müller,* and Andreas Herrmann*
*Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, D-10115 Berlin, Germany; #Technische Universität Berlin, Max-Volmer-Institut für Biophysikalische Chemie und Biochemie, D-10623 Berlin, Germany; and §Department of Chemistry, Wichita State University, Wichita, Kansas 67260 USA

ABSTRACT Spin-labeled phospholipid analogs have been employed to probe the transbilayer distribution of endogenous phospholipids in various membrane systems. To determine the transmembrane distribution of the spin-labeled analogs, the analogs are usually inserted into the membrane of interest and subsequently the amount of analog in the outer membrane leaflet is determined either by chemical reduction with ascorbate or by back-exchange to bovine serum albumin (BSA). For accurate determination of the transbilayer distribution of analogs, both the kinetics of incorporation and those of accessibility of analogs to ascorbate or BSA have to be fast in comparison to their transbilayer movement. By means of stopped-flow electron paramagnetic resonance (EPR) spectroscopy, we have studied the kinetics of incorporation of the spin-labeled phosphatidylcholine (PC) analog 1-palmitoyl-2-(4-doxylpentanoyl)-sn-glycero-3-phosphocholine (SL-PC) and of its accessibility to chemical reduction and to back-exchange at room temperature. Incorporation of SL-PC into the outer leaflet of egg phosphatidylcholine (EPC) and red cell ghost membranes was essentially completed within 5 s. Ninety percent of the SL-PC molecules located in the outer membrane leaflet of those membranes were extracted by BSA within 15 s. All exterior-facing SL-PC molecules were reduced by ascorbate in a pseudo-first-order reaction within 60 s in EPC membranes and within 90 s in red cell ghost membranes. The rate of the reduction process could be enhanced by ~30-fold when 6-*O*-phenyl-ascorbic acid was used instead of ascorbate as the reducing agent. The results are discussed in light of assaying rapid transbilayer movement of spin-labeled analogs in biological membranes.

INTRODUCTION

Since the early work of Devaux and co-workers on the human erythrocyte membrane (Seigneuret and Devaux, 1984; Seigneuret et al., 1984), spin-labeled phospholipids have become an important tool for the determination of transverse phospholipid distribution in biological membranes (for recent reviews see, e.g., Zachowski, 1993, or Menon, 1995). The phospholipid analogs developed for those studies carry a long-chain fatty acid at the sn-1 position and a short spin-labeled fatty acid at the sn-2 position (Fig. 1) (Seigneuret et al., 1984; Fellmann et al., 1994). In the absence of membranes, those analogs form micelles in aqueous solutions with a critical micellar concentration of ~1 μM (King and Marsh, 1987). Although this behavior is similar to that of lysolipids, numerous studies on a variety of plasma membranes and subcellular membranes have demonstrated that spin-labeled phospholipids are quite faithful analogs of their biogenic counterparts (Zachowski et al., 1987; Calvez et al., 1988; Morrot et al., 1989; Wu and Hubbell, 1993), whereas spin-labeled lysolipids are not (Morrot et al., 1989). As long as the membrane concentration of spin-labeled phospholipids (Fig. 1) is low (<5 mol% of endogenous lipids), cellular viability does not seem to be impaired. For example, we have recently shown that the motility of ram sperm cells was not affected after incorporation of analogs into the plasma membrane (Müller et al., 1994).

The basic principle for probing the transbilayer distribution of spin-labeled phospholipid analogs is rather simple. In aqueous buffer these analogs form a suspension with coexisting populations of monomers and micelles. When mixed with native or artificial membranes these spin-labeled phospholipids partition quantitatively into that leaflet of a membrane bilayer that is exposed to the buffer (hereafter called the outer leaflet). Subsequently, the lipid analogs may redistribute between the outer and inner leaflet by various pathways, e.g., passive and/or ATP-dependent mechanisms (Zachowski, 1993; Menon, 1995). As has been shown for plasma membranes and subcellular membranes of various cell types (see references above), the analogs finally adopt a transmembrane distribution that is very close to the distribution observed for endogenous phospholipids. In principle, two experimental approaches for determining the transbilayer distribution of spin-labeled phospholipids have found widespread application. One way is to treat labeled membranes with membrane-impermeant reagents such as ascorbate to selectively reduce the NO moiety of the spin-labeled analogs located in the outer membrane leaflet to the corresponding nonparamagnetic hydroxylamine. This so-called ascorbate assay was originally worked out by

Received for publication 20 March 1995 and in final form 12 June 1997.
Address reprint requests to Dr. Andreas Herrmann, Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, Invalidenstrasse 43, D-10115 Berlin, Germany. Tel.: 49-30-20938830; Fax: 49-30-20938585; E-mail: andreas=herrmann@rz.hu-berlin.de.

0006-3495/97/09/1645/10 $2.00

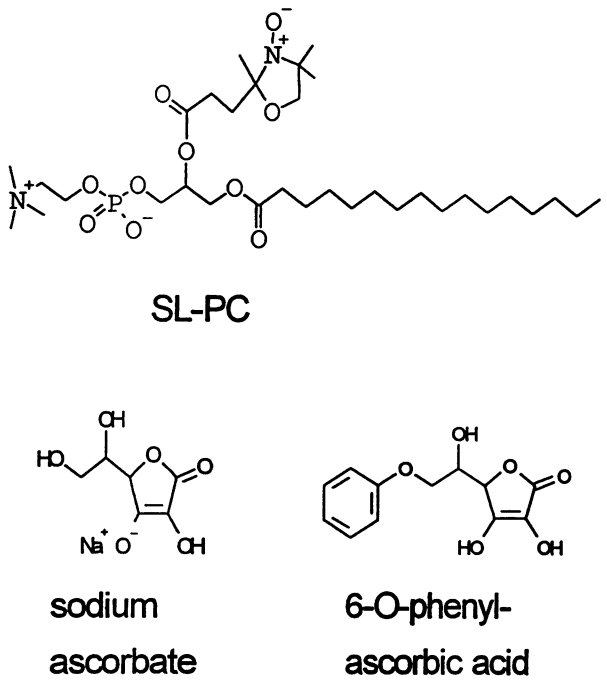

FIGURE 1 Structures of 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine, sodium ascorbate, and of 6-*O*-phenyl-ascorbic acid.

Kornberg and McConnell (1971) for a spin-labeled phosphatidylcholine (PC) analog with the paramagnetic tetramethylpiperidine-1-oxyl group covalently bonded to the quaternary ammonium group of choline. A prerequisite for efficient reduction by ascorbate is that the NO group be located close to the lipid-aqueous interface. The structure of the phospholipid analog shown in Fig. 1 meets this condition (Seigneuret and Devaux, 1984; Seigneuret et al., 1984).

Alternatively, the population of spin label on the exterior may be ascertained by back-exchange of exterior-facing spin-labeled phospholipid analogs to bovine serum albumin (BSA) (Calvez et al., 1988; Morrot et al., 1989). This "back-exchange" assay was originally developed for extraction of lysolipids and fatty acids from membranes (Haest et al., 1981).

The time resolution of the ascorbate and the back-exchange assay depends 1) on the rate of the incorporation of the spin-labeled phospholipid analogs into membranes and 2) on the rate of the reduction (in the case of the ascorbate assay) or the extraction (in the case of the back-exchange assay) of the analogs that reside in the outer membrane leaflet. Recent work on the rapid translocation of phospholipids across the plasma membrane of sperm cells (Müller et al., 1994) and the membrane of rat liver endoplasmic reticulum (Buton et al., 1996) clearly demonstrates the need for assaying the transbilayer phospholipid distribution on a rapid time scale. Unfortunately, quantitative data characterizing the time dependence of the incorporation and the accessibility of spin-labeled phospholipid analogs are not available.

In the present study we were able to define an upper limit for the time resolution that is achievable with the back-exchange and the ascorbate assay, by employing stopped-flow electron paramagnetic resonance (EPR) spectroscopy with a dead time of 10 ms. We have determined the kinetics 1) of the incorporation of the spin-labeled analog 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine (SL-PC) into liposomal and biological membranes, 2) of the back-exchange of SL-PC from the outer bilayer leaflet to BSA, and 3) of the reduction of the NO moiety of SL-PC localized in the outer leaflet of liposomal and biological membranes by ascorbate. To optimize the latter approach we examined various ascorbate derivatives as reducing agents and found that the rate of the reduction process can be significantly enhanced by using 6-*O*-phenyl-ascorbic acid instead of the usual ascorbate.

MATERIALS AND METHODS

Chemicals

Egg phosphatidylcholine (EPC), KCl, $MgCl_2$, NaCl, Na_2HPO_4, NaH_2PO_4, sodium ascorbate, and BSA were purchased by Sigma (Deisenhofen, Germany). Tris was obtained from Fluka (Neu-Ulm, Germany). Phosphate-buffered saline (PBS) contained 150 mM NaCl and 5.8 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4).

Synthesis of SL-PC and 6-*O*-phenyl-ascorbic acid

SL-PC and 6-*O*-phenyl-ascorbic acid (Fig. 1) were synthesized as described previously (Fellmann et al., 1994; Wimalasena et al., 1994).

Preparation of unlabeled small unilamellar phospholipid vesicles

An appropriate amount of EPC dissolved in chloroform/methanol (9:1, v/v) was dried under nitrogen and resuspended at a concentration of 4 mM in low salt buffer (LSB) (50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5). The resulting phospholipid dispersion was sonicated for 30 min with the microtip of a Branson Sonifier model W 250 (Carouge-Geneve, Switzerland) at an output control setting of 2 and 50% duty cycle in a glass tube cooled in an ice bath.

Preparation of spin-labeled small unilamellar vesicles

Small unilamellar vesicles (SUVs) containing SL-PC in both leaflets of the membrane bilayer (symmetrically labeled vesicles) were prepared by mixing 2 μmol of EPC with 0.1 μmol of SL-PC, all dissolved in chloroform/methanol (9:1, v/v). The phospholipid mixture was dried under nitrogen, and the phospholipids were resuspended in 1 ml of LSB. Symmetrically labeled SUVs were made by sonicating the resulting phospholipid dispersion for 30 min on ice as already described for unlabeled small unilamellar EPC vesicles (see above). SUVs containing SL-PC only in the outer leaflet of the membrane bilayer (asymmetrically labeled vesicles) were made by preparing unlabeled small unilamellar EPC vesicles (4 mM EPC) as described above and by mixing them with an equal volume of 0.2 mM SL-PC in LSB.

Preparation of human erythrocyte ghosts

Unsealed erythrocyte ghosts were prepared according to the method of Dodge et al. (1963). After removal of plasma and buffy coat, erythrocytes

were washed twice in PBS at 2000 × *g* and finally resuspended to a 50% hematocrit. Lysis was performed at 4°C by dilution of 4 ml ice-cold suspension of erythrocytes (50% packed) with 60 ml ice-cold lysis buffer (5.8 mM NaH_2PO_4/Na_2HPO_4, pH 7.4). Unsealed ghosts were collected by centrifugation at 25,000 × *g* for 10 min and washed with ice-cold PBS. Concentration of ghost proteins was determined according to the method of Lowry et al. (1951). A weight ratio of ghost proteins to ghost endogenous phospholipids of 1:1 was assumed.

Stopped-flow experiments

All experiments were carried out at room temperature with a commercially available EPR stopped-flow accessory (Center of Scientific Instruments of the former Academy of Sciences, Berlin-Adlershof) attached to a X-band EPR spectrometer (type ERS 300 E; Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany). The following parameters were always used: power 20 mW, modulation frequency 100 kHz, modulation amplitude ≤4 G. The stopped-flow accessory was specifically designed for use in EPR spectroscopy, meeting the typical demands of biological applications, e.g. small consumption of substance, avoidance of any contact of the reactants with metal, and use of aqueous systems (Klimes et al., 1980). For commercial production this original laboratory device was improved by a pressure-resistant cylindrical capillary EPR cell and by the installation of a stopping valve. This device has also been applied in enzymology, e.g., for detection and characterization of a transient inhibitor radical of ribonucleotide reductase (Lassmann et al., 1992). The EPR cell is a thick-walled cylindrical quartz capillary with a 1.3-mm inner diameter (sample volume: 30 μl) connected immediately with the mixer. The cylindrical capillary EPR cell allows the connection of the stopped-flow accessory to any rectangular standard H_{102}-X-band cavity with a 11-mm sample hole. One mixing shot needs 70 μl of each of the liquid components, which are stored in glass syringes (1.6 ml each). Stroking magnets drive the two components through the mixer and the EPR cell. Behind the EPR cell the flow is stopped by an electrically controlled stopping valve, setting the trigger to record a time sweep. The mixing takes ~ 1 ms and the dead time (time between mixing and entering the EPR cell) another 1 ms. Although the stopping time is only 0.5 ms, the stopping valve causes mechanical disturbances that have an impact on the microwave detection system and last some milliseconds. The overall time resolution of the apparatus has been measured to be 10 ms, that is 10 ms after the trigger is set to record a time sweep, the EPR signal is free of any disturbance from the stopped-flow accessory.

Incorporation of SL-PC into membranes

To measure the time-dependent incorporation of SL-PC into membranes, SL-PC was resuspended at a concentration of 0.2 mM in LSB and mixed with an equal volume of a suspension of unlabeled EPC SUVs (4 mM EPC) or an equal volume of a ghost suspension (4 mM ghost phospholipid) in the stopped-flow accessory. The kinetics of incorporation was followed by measuring the change of the EPR amplitude at a position corresponding to the maximum of the high-field peak of the SL-PC membrane spectrum (see also Results, Fig. 2). Kinetics in Fig. 3 were fitted to the following biexponential function:

$$I_{EPR} = a * (1 - e^{-k_1 * t}) + b * (1 - e^{-k_2 * t})$$

where t is the time and I_{EPR} is the signal intensity, which is proportional to the amount of incorporated SL-PC (see below).

Accessibility of membrane-incorporated SL-PC to BSA, ascorbate, and 6-*O*-phenyl-ascorbic acid

BSA extraction of SL-PC from asymmetrically labeled EPC vesicle membranes or from red cell ghost membranes was monitored by mixing asymmetrically labeled EPC vesicles (2 mM EPC, 0.1 mM SL-PC) or labeled ghosts (2 mM ghost phospholipid, 0.1 mM SL-PC) with an equal volume of 10% (w/v) BSA in LSB in the stopped-flow cell. The time-dependent decrease of the EPR amplitude at a position corresponding to the maximum of the low-field peak of the SL-PC membrane spectrum (see also Results, Figs. 7 and 8) reflects the kinetics of BSA extraction. Reduction of the EPR signal of SL-PC in spin-labeled ghosts or in symmetrically or asymmetrically labeled EPC vesicles due to ascorbate or 6-*O*-phenyl ascorbic acid was monitored by mixing a suspension of labeled ghosts (2 mM ghost phospholipid, 0.1 mM SL-PC) or a suspension of the respective labeled vesicles (2 mM EPC, 0.1 mM SL-PC) with an equal volume of 100 mM sodium ascorbate or 20 mM 6-*O*-phenyl ascorbic acid in LSB in the stopped-flow cell. The time-dependent decrease in EPR amplitude at a position corresponding to the maximum of the low-field peak (see also Results, Figs. 4 and 5) was taken as a measure of SL-PC reduction. 6-*O*-Phenyl-ascorbic acid is considerably more acidic than sodium ascorbate. To preclude any influence of the pH on the rate of spin-label reduction, 6-*O*-phenyl ascorbic acid solutions were adjusted to a pH of 7.5 with sodium hydroxide before the experiments.

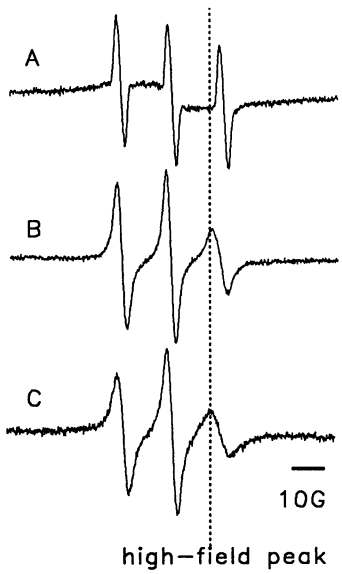

FIGURE 2 Incorporation of SL-PC into EPC vesicle membranes and red cell ghost membranes. The top spectrum (*A*) is the spectrum of SL-PC in aqueous buffer. Spectrum *B* was recorded after quantitative incorporation of SL-PC into EPC vesicle membranes (molar ratio SL-PC:EPC = 5:100). Spectrum *C* was recorded after incorporation of SL-PC into red cell ghost membranes (molar ratio SL-PC to ghost phospholipids = 5:100). All spectra were recorded at room temperature. The specific parameters of EPR measurement were: conversion time, 81.92 ms (*A*, *B*), 163.64 ms (*C*); time constant, 20.48 ms (*A*, *B*), 40.96 ms (*C*).

RESULTS

Time course of SL-PC incorporation into EPC vesicle membranes and red cell ghost membranes

The EPR spectrum of SL-PC in buffer is shown in Fig. 2 *A*. It is a composite spectrum consisting of a broad component and a very narrow triplet. The broad component is due to strong spin-spin interaction arising from SL-PC organized in micelles. The narrow triplet is caused by SL-PC monomers rapidly tumbling in buffer. Upon the addition of aqueous dispersed SL-PC to unlabeled EPC vesicles or red cell

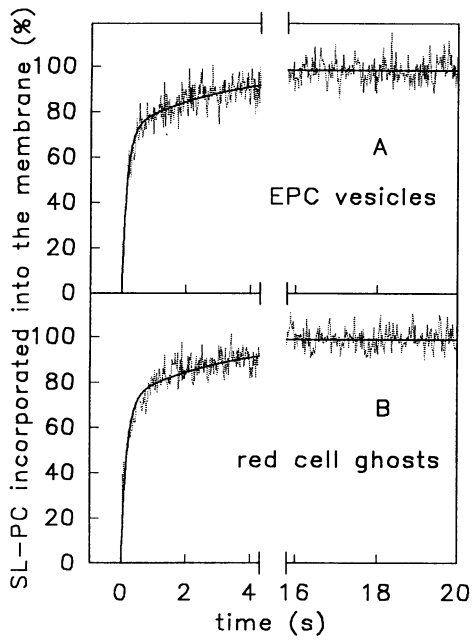

FIGURE 3 (*Curve A*) Kinetics of the incorporation of SL-PC into EPC vesicle membranes at room temperature (molar ratio SL-PC:EPC = 5:100). The solid line was obtained by fitting the data to a biexponential function (see Materials and Methods) with the following parameters: a = 71.8%, b = 26.4%, k_1 = 6.21 s^{-1}, k_2 = 0.32 s^{-1}. (*Curve B*) Kinetics of the incorporation of SL-PC into red cell ghost membranes at room temperature (molar ratio SL-PC to ghost phospholipids = 5:100). The solid line was obtained by fitting the data to a biexponential function (see Materials and Methods) with the following parameters: a = 72.3%, b = 26.6%, k_1 = 5.63 s^{-1}, k_2 = 0.30 s^{-1}. The specific parameters of EPR measurement were (*A*, *B*): conversion time, 20.48 ms; time constant, 5.12 ms. The time interval between two data points is 20.48 ms in both kinetics.

ghosts (molar ratio SL-PC to EPC or ghost phospholipids = 5:100), all analogs became incorporated into the membrane, as indicated by a typical membrane spectrum (Fig. 2, *B* and *C*).

To monitor the kinetics of incorporation of analogs into EPC vesicle membranes or ghost membranes by EPR stopped-flow spectroscopy, we set the magnetic field to a position corresponding to the maximum of the high-field peak of the membrane spectrum (see *marking line* in Fig. 2). A comparison between the SL-PC membrane spectra (Fig. 2, *B* and *C*) and the spectrum of the analog in aqueous buffer (Fig. 2 *A*) revealed that at this field strength the incorporation of SL-PC into EPC or ghost membranes was accompanied by a significant change in the EPR amplitude. Thus the kinetics of incorporation of SL-PC could be measured by monitoring the increase in amplitude as a function of time. The result of such a stopped-flow experiment performed with SL-PC and EPC vesicles is shown in Fig. 3 *A*. An analogous experiment is shown with ghosts in Fig. 3 *B*. Equal volumes of an aqueous SL-PC dispersion and a suspension of EPC vesicles or ghosts were rapidly mixed and injected into the EPR stopped-flow cell (see Materials and Methods). From Fig. 3, *A* and *B*, it can be deduced that 90% of the SL-PC analogs became incorporated into EPC

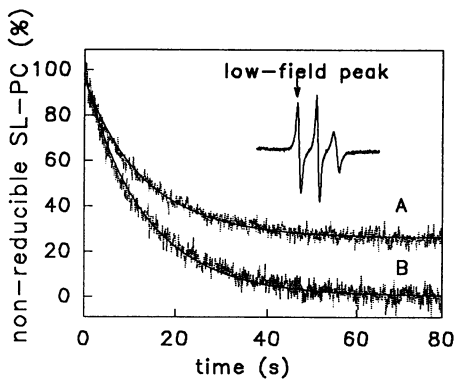

FIGURE 4 (*Curve A*) Kinetics of the reduction of SL-PC in symmetrically labeled EPC vesicles (molar ratio SL-PC:EPC = 5:100) by 50 mM sodium ascorbate (final concentration) at room temperature. The solid line was obtained by fitting the data to Eq. 1. (*Curve B*) Kinetics of the reduction of SL-PC in asymmetrically labeled EPC vesicles (molar ratio SL-PC:EPC = 5:100) by 50 mM sodium ascorbate (final concentration) at room temperature. The solid line was obtained by fitting the data to Eq. 2. The specific parameters of EPR measurement were (*A*, *B*): conversion time, 81.92 ms; time constant, 40.96 ms. The time interval between two data points is 81.92 ms in both kinetics. (*Inset*) Spectrum of SL-PC incorporated into the membrane of EPC vesicles (molar ratio SL-PC: EPC = 5:100).

vesicle membranes and ghost membranes within 5 s at room temperature. Kinetics could be fitted by a biexponential function (see Materials and Methods as well as legend to Fig. 3) with rate constants on the order of 6 s^{-1} and 0.3 s^{-1}, respectively. The corresponding amplitudes were ~72% and 27%, respectively. After the incorporation kinetics of SL-PC was measured, its EPR spectrum was recorded directly in the stopped-flow cell. Both in the case of EPC vesicles and in the case of ghosts a typical membrane spectrum was found, confirming the complete incorporation of the analogs into EPC and ghost membranes.

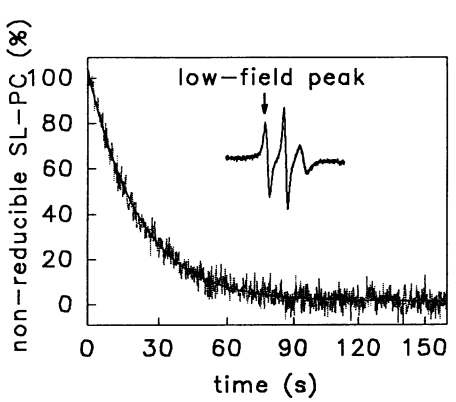

FIGURE 5 Kinetics of the reduction of SL-PC in red cell ghost membranes (molar ratio SL-PC to ghost phospholipids = 5:100) by 50 mM sodium ascorbate (final concentration) at room temperature. The specific parameters of EPR measurement were: conversion time, 163.84 ms; time constant, 40.96 ms. The solid line was obtained by fitting the data to Eq. 2. The time interval between two data points is 163.84 ms. (*Inset*) Spectrum of SL-PC incorporated into the membrane of red cell ghosts (molar ratio SL-PC to ghost phospholipids = 5:100).

To verify that the measured kinetics indeed reflects the incorporation of the analog into membranes but not alterations due to technical limitations of the stopped-flow equipment (such as mixing time, mechanical disturbance, etc.; see Materials and Methods), we have recorded the time-dependent change of the high-field peak amplitude upon mixing of SL-PC suspension with buffer (dilution, no vesicles). In that case alterations of EPR amplitude were found only for $t < 10$ ms, whereas for later times the amplitude remained constant (data not shown).

Reduction of SL-PC in EPC membranes by ascorbate

EPC vesicles labeled exclusively on their outer leaflet with SL-PC (subsequently called "asymmetrically labeled") were prepared by mixing SL-PC in buffer with unlabeled EPC vesicles (see Materials and Methods). In phospholipid membranes transbilayer diffusion of phospholipids such as PC is very slow, with half-times on the order of hours or days at room temperature (Op den Kamp, 1979). Indeed, our data confirm that in asymmetrically labeled vesicles all SL-PC molecules remained in the outer leaflet during the time course of our experiments. We have mixed asymmetrically labeled EPC vesicles with an equal volume of 100 mM ascorbate in the EPR stopped-flow cell (see Materials and Methods) and have followed the amplitude of the low-field peak (see *arrow* in the *inset* of Fig. 4) as a function of time at room temperature. Because ascorbate reduces the NO moiety of SL-PC to the corresponding nonparamagnetic hydroxylamine, the low-field peak amplitude decreases and, in the case of asymmetrically labeled vesicles, eventually disappears. Fig. 4 *B* displays the time-dependent decrease of the low-field peak EPR amplitude for asymmetrically labeled EPC vesicles. After recording of the kinetics, the EPR spectrum of SL-PC could no longer be detected, confirming the complete reduction of the analog by ascorbate (data not shown). This is consistent with the SL-PC being localized exclusively in the outer leaflet of asymmetrically labeled EPC vesicles. Additional support for that conclusion is given by experiments on "symmetrically labeled" vesicles (see below).

The kinetics of the reduction process can be described by a reaction that is first order in both SL-PC and ascorbate (see also Wu and Hubbell, 1993). The concentration of nonreduced label at a given time is $[N_{tot}]$ and corresponds to the sum of intact label on the outer $[N_0]$ and inner $[N_i]$ leaflet. At the time of ascorbate addition ($t = 0$ s), $[N_{tot}]_{t=0}$ is 100%. If one assumes the SL-PC that is located in the inner vesicle leaflet is inaccessible to ascorbate, the relevant rate equation is

$$[N_{tot}] = ([N_{tot}]_{t=0} - [N_i])e^{-k_r[asc_0]t} + [N_i] \quad (1)$$

where $[asc_0]$ is the concentration of ascorbate on the exterior of the vesicle and k_r is the rate constant for the reduction. In the case of asymmetrically labeled vesicles ($[N_i] = 0\%$), the rate equation simplifies to

$$[N_{tot}] = [N_{tot}]_{t=0}e^{-k_r[asc_0]t} \quad (2)$$

The concentration of ascorbate $[asc_O]$ is in large excess over the concentration of SL-PC and may therefore be taken to be constant. Thus the reduction process is pseudo-first-order with respect to SL-PC, and the reduction of curve *B* in Fig. 4 can be fit well by a single exponential. The half-time $t_{1/2}$ is given by

$$t_{1/2} = \frac{1}{k_r[asc_0]} \ln 2 \quad (3)$$

At room temperature, $t_{1/2}$ of the reduction of SL-PC in asymmetrically labeled EPC was 9.7 s at 50 mM ascorbate (final concentration), and the respective rate constant k_r was 1.42 M^{-1} s^{-1} (see also Table 1).

For comparison, "symmetrically labeled" EPC vesicles were made by including SL-PC during vesicle preparation (see Materials and Methods). Under this condition, the distribution of the paramagnetic analog mimics the transbilayer distribution of EPC across the vesicle bilayer. Curve *A* in Fig. 4 shows the reduction of SL-PC in symmetrically labeled EPC vesicles after they are mixed with an equal volume of 100 mM ascorbate in the stopped-flow accessory at room temperature as described above. In the case of symmetrically labeled vesicles, the amplitude of the low-field peak did not decay to zero, as in the case of asym-

TABLE 1 Reduction of SL-PC in the outer leaflet of EPC vesicles and red cell ghost membranes by ascorbate and 6-*O*-phenyl-ascorbic acid at room temperature

Membrane	$[N_i]$ (%)	Reducing agent	$[asc_0]$ (mM)	$t_{1/2}$ (s)	k_r ($M^{-1}s^{-1}$)
EPC vesicles (asymmetrical)	0	Ascorbate	50	9.7 ± 0.1	1.42 ± 0.02
EPC vesicles (symmetrical)	28 ± 3	Ascorbate	50	9.5 ± 0.4	1.46 ± 0.06
Red cell ghosts (asymmetrical)	ND	Ascorbate	50	14.5 ± 0.5	0.96 ± 0.03
EPC vesicles	0	6-*O*-Phenyl-ascorbic acid	10	1.6 ± 0.1	42.49 ± 0.43

Measurements were performed by means of a stopped-flow accessory attached to an EPR spectrometer as described in Materials and Methods. $[asc_0]$ corresponds to the concentration of the reducing agent. The reduction rate constants k_r (see Eqs. 1 and 2) and the half-times $t_{1/2}$ of reduction (see Eq. 3) were obtained by fitting the experimental kinetics to Eq. 1 (symmetrically labeled vesicles) or Eq. 2 (asymmetrically labeled vesicles). Data represent the average ± SD of three independent experiments.

metrically labeled vesicles (see Fig. 4, *curve B*), but remained constant at a level of 28% of the initial label concentration $[N_{tot}]_{t=0}$. After reaching the plateau, we have followed the remaining EPR spectrum for an additional 5 min at room temperature. We found no further decrease in intensity. Such a stable, time-independent plateau value is consistent with a negligible permeability of EPC vesicles for ascorbate, even at room temperature.

One may wonder whether part of the vesicles became (transiently) leaky because of mechanical stress by shearing forces during rapid mixture in the EPR stopped-flow accessory, which could cause a partial reduction of SL-PC in the inner monolayer. This can obviously be ruled out, because we obtained the same fraction of nonreducible SL-PC when symmetrically labeled vesicles were gently mixed with ascorbate and then transferred to a conventional EPR quartz capillary. Therefore, the observed plateau value of 28% of the initial label concentration can be attributed to SL-PC that is located in the inner monolayer of the EPC vesicle membranes. We fit the reduction kinetics in Fig. 4 *A* to Eq. 1. $[N_{tot}]$ is 100% at the time of ascorbate addition. $[N_i]$ = 28% is given by the plateau value in Fig. 4 *A*, and, as the ascorbate penetration through EPC bilayers is negligibly small at room temperature, $[N_i]$ can be treated as a constant. As expected, we obtained almost the same values for the half-time $t_{1/2}$ and for the rate constant k_r of reduction of SL-PC in the outer monolayer by 50 mM ascorbate (final concentration) as for asymmetrically labeled vesicles ($t_{1/2}$ = 9.5 s, k_r = 1.46 M^{-1} s^{-1}; see also Table 1).

Reduction of SL-PC in red cell ghost membranes

We have measured the SL-PC reduction kinetics in unsealed human erythrocyte ghosts. After labeling with SL-PC, the ghosts were mixed with an equal volume of 100 mM ascorbate in the stopped-flow cell at room temperature as described above. Fig. 5 shows the time-dependent reduction of SL-PC in ghost membranes by ascorbate. All SL-PC molecules were accessible to ascorbate. The latter was confirmed in that no EPR signal could be detected when the EPR spectrum was recorded a few minutes after the ascorbate treatment (data not shown). The kinetics can be fit well by Eq. 2. This suggests that all SL-PC molecules were oriented to the exoplasmic leaflet. Indeed, the transbilayer movement of SL-PC in red blood cell membranes is a very slow, passive process (Seigneuret and Devaux, 1984; Morrot et al., 1989). We cannot rule out the possibility that holes of leaky ghosts may provide a site of accelerated transbilayer movement, and thus a small amount of SL-PC may have redistributed to the inner leaflet. However, in that case it is reasonable to assume that ascorbate could rapidly penetrate across leaky ghost membranes, reducing SL-PC in the inner leaflet with the same efficiency as in the outer leaflet. Otherwise, the reduction reaction should not be describable by a monoexponential function. We found that the reduction of SL-PC in red cell ghost membranes was slower than in EPC vesicle membranes ($t_{1/2}$ = 14.5 s compared to, e.g., $t_{1/2}$ = 9.7 s in asymmetrically labeled EPC vesicles; see also Table 1). The rate constant k_r for the reduction of SL-PC in ghost membranes was 0.96 M^{-1} s^{-1} (see also Table 1).

Reduction of SL-PC in EPC membranes by 6-*O*-phenyl-ascorbic acid

Fig. 6 (*curve B*) shows the kinetics of reduction of EPC vesicles asymmetrically labeled with SL-PC by 6-*O*-phenyl-ascorbic acid. Equal volumes of asymmetrically labeled EPC vesicles and 20 mM 6-*O*-phenyl-ascorbic acid were mixed in the stopped-flow cell, and the decrease in the low-field peak amplitude was followed as a function of time at room temperature as described above. We found that the reduction of SL-PC by 6-*O*-phenyl ascorbic acid was much faster in comparison to that by ascorbate, even at a fivefold lower concentration (10 mM final concentration in the case of 6-*O*-phenyl-ascorbic acid compared to 50 mM final concentration in the case of ascorbate; see Materials and Methods). In less than 10 s, the EPR signal of SL-PC disappeared completely, which was confirmed by recording a total spectrum a few minutes after the addition of 6-*O*-phenyl-ascorbic acid. The reduction was pseudo-first-order with respect to SL-PC, and the reduction curve could be fit by a single exponential according to Eq. 2 with a half-time $t_{1/2}$ of 1.6 s at room temperature (see also Table 1). The rate constant k_r for the reduction of asymmetrically labeled vesicles with 6-*O*-phenyl-ascorbic acid (k_r = 42.49 M^{-1} s^{-1}) was ~30-fold higher in comparison to k_r for the reduction of asymmetrically labeled vesicles with ascorbate (k_r = 1.42 M^{-1} s^{-1}).

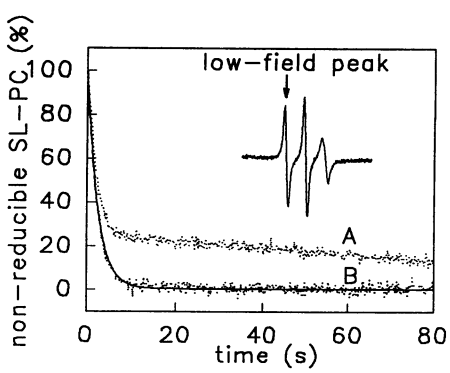

FIGURE 6 (*Curve A*) Kinetics of the reduction of SL-PC in symmetrically labeled EPC vesicles (molar ratio SL-PC:EPC = 5:100) by 10 mM 6-*O*-phenyl-ascorbic acid (final concentration) at room temperature. (*Curve B*) Kinetics of the reduction of SL-PC in asymmetrically labeled EPC vesicles (molar ratio SL-PC:EPC = 5:100) by 10 mM 6-*O*-phenyl-ascorbic acid (final concentration) at room temperature. The solid line was obtained by fitting the data to Eq. 2. The specific parameters of EPR measurement were (*A*, *B*): conversion time, 163.84 ms; time constant, 40.96 ms. The time interval between two data points is 163.84 ms in both kinetics. (*Inset*) Spectrum of SL-PC incorporated into the membrane of EPC vesicles (molar ratio SL-PC:EPC = 5:100).

Fig. 6 (*curve A*) shows the disappearance of the EPR signal of SL-PC in symmetrically labeled EPC vesicles in the presence of 10 mM 6-*O*-phenyl-ascorbic acid (final concentration) at room temperature. Again, we found a rapid decay of the EPR signal corresponding to reduction of SL-PC located in the outer leaflet. However, in contrast to similar experiments with ascorbate (see Fig. 4 *A*), we did not observe a stable plateau corresponding to the SL-PC on the inner leaflet, but a further, slower continuous, and, finally, complete loss of the EPR signal. We surmise that at room temperature, 6-*O*-phenyl-ascorbic acid penetrates slowly through the bilayer of EPC vesicles, causing the slow reduction of SL-PC located in the intravesicular leaflet. By extrapolation of the second slow rate component to zero time, it could be shown that ~26% of the SL-PC are located in the inner leaflet of symmetrically labeled EPC vesicles. This value is consistent with that obtained from the data in Fig. 4 *A*. We could demonstrate by conventional EPR spectroscopy (without stopped-flow mixing) that the permeation of 6-*O*-phenyl-ascorbic acid was strongly temperature dependent. At 0°C 6-*O*-phenyl-ascorbic acid did not penetrate through EPC vesicle bilayers, and a stable plateau corresponding to SL-PC analogs on the outer leaflet was observed (data not shown). Even at this low temperature, reduction of SL-PC by 6-*O*-phenyl-ascorbic acid was too rapid to resolve into the corresponding kinetics with conventional EPR spectroscopy.

Extraction of SL-PC from the outer membrane leaflet of EPC vesicles and red cell ghosts

The EPR spectrum of SL-PC incorporated into the outer leaflet of asymmetrically labeled EPC vesicles is shown in Fig. 7 *A*. Upon mixing of these vesicles with an equal volume of 10% (w/v) BSA, the spectrum in Fig. 7 *B* was obtained. As mixing of SL-PC in buffer with 10% (w/v) BSA (data not shown) yielded a spectrum that is identical to the spectrum in Fig. 7 *B*, we conclude that all SL-PC molecules initially located in the outer leaflet of asymmetrically labeled vesicles were extracted by BSA. We have performed the same set of experiments with red cell ghosts and we obtained analogous results. Upon mixing of ghosts labeled with SL-PC with 10% (w/v) BSA, all SL-PC molecules located in the red cell ghost membranes were extracted by BSA (data not shown). Because the spectrum of SL-PC bound to BSA is more immobilized in comparison to the typical SL-PC membrane spectra obtained with ghosts or EPC vesicles (see Fig. 7, *A* and *B*, data for ghosts not shown), a decrease in the EPR amplitude of the low-field peak (see *marking line*, Fig. 7) is observed upon back-exchange of SL-PC from EPC vesicle or ghost membranes to BSA. Thus the kinetics of extraction of SL-PC from the outer leaflet of EPC vesicle or ghost membranes could be determined by mixing EPC vesicles asymmetrically labeled with SL-PC or ghosts labeled with SL-PC with an equal volume of 10% (v/w) BSA in the stopped-flow cell and monitoring the time-dependent decrease in the low-field peak amplitude at room temperature (see Materials and Methods). Fig. 8 displays the kinetics of extraction of SL-PC from the outer membrane leaflet of asymmetrically

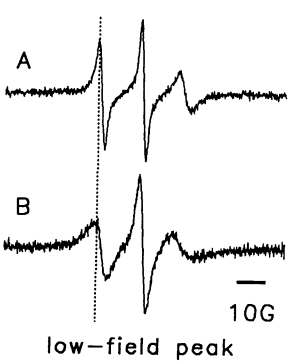

FIGURE 7 Extraction of SL-PC from the outer leaflet of asymmetrically labeled EPC vesicles (molar ratio SL-PC:EPC = 5:100) by BSA. The top spectrum (*A*) was recorded after quantitative incorporation of SL-PC into the outer leaflet of EPC vesicles (conversion time, 81.92 ms; time constant, 20.48 ms; time interval between two data points, 81.92 ms). The bottom spectrum (*B*) was recorded after quantitative extraction of the SL-PC from the outer membrane leaflet of the above-mentioned EPC vesicles by 5% (w/v) BSA (final concentration) (conversion time, 163.84 ms; time constant, 40.96 ms; time interval between two data points, 163.84 ms). Both spectra were recorded at room temperature.

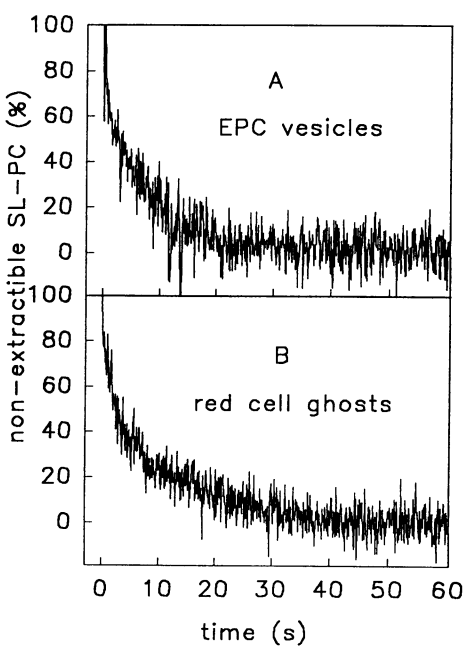

FIGURE 8 (*Curve A*) Kinetics of the extraction of SL-PC from the outer membrane leaflet of asymmetrically labeled EPC vesicles (molar ratio SL-PC:EPC = 5:100) by 5% (w/v) BSA (final concentration) at room temperature. (*Curve B*) Kinetics of the extraction of SL-PC from red cell ghost membranes (molar ratio SL-PC to ghost phospholipids = 5:100) by 5% (w/v) BSA (final concentration) at room temperature. The specific parameters of EPR measurement were (*A*, *B*): conversion time, 81.92 ms; time constant, 40.96 ms. The time interval between two data points is 81.92 ms in both kinetics.

labeled EPC vesicles (*curve A*) or from the membrane of labeled ghosts (*curve B*). In both EPC and ghost membranes, 90% of the SL-PC molecules were extracted by BSA within 15 s at room temperature.

DISCUSSION

Spin-labeled phospholipid analogs with a long fatty acid chain in the sn-1 position and a short-chain fatty acid bearing the NO moiety in the sn-2 position have been successfully employed for investigating the transbilayer motion and distribution of phospholipids in plasma and subcellular membranes of various mammalian cells. Similar phospholipid analogs with the fluorescent 4-nitrobenzo-2-oxa-1,3-diazole group attached to the short fatty acid chain have become an important tool for studying phospholipid dynamics by fluorescence microscopy and spectroscopy (Martin and Pagano, 1987; Connor et al., 1990).

Different assays have been employed to measure the transbilayer redistribution of spin-labeled phospholipid analogs. In particular, the ascorbate assay and the back-exchange assay have been established for measuring the transmembrane distribution in a variety of plasma and subcellular membranes. Devaux (1991) has pointed out that the steady-state transmembrane distribution that is observed for phospholipid analogs results from a subtle balance between analogs redistributing from the outer to the inner membrane leaflet and vice versa. Thus both the incorporation of spin-labeled analogs into the membrane and the assay reactions for determining their transmembrane distribution have to be faster than the above-mentioned inward and outward movements. Whereas these preconditions seem to be fulfilled for the plasma membrane of a variety of mammalian cells such as erythrocytes (Seigneuret and Devaux, 1984; Calvez et al., 1988) or fibroblasts (Pomorski et al., 1996), we have shown recently that the rate of the aminophospholipid translocase-mediated translocation of spin-labeled aminophospholipid analogs from the exoplasmic to the cytoplasmic leaflet of the plasma membrane of ram sperm cells is close to the time resolution of the back-exchange assay (Müller et al., 1994). Moreover, the transbilayer movement of phospholipid analogs in various subcellular membranes can be very fast, with half-times on the order of minutes (Wu and Hubbell, 1993) or even less than 1 min. For instance, Dolis et al. (1996) have reported that radioactively labeled PC redistributes across the outer membrane of rat liver mitochondria with a half-time of 2 min at 30°C. Buton et al. (1996) have reported that spin-labeled phospholipid analogs redistribute across the membrane of the endoplasmic reticulum of rat liver with a half-time of 25 s at 20°C (PC and phosphatidylserine) and 20 s at 20°C (phosphatidylethanolamine). To our knowledge this is the fastest translocation of spin-labeled analogs observed so far in biological membranes. A main prerequisite for the validity of the above-mentioned studies is that 1) the incorporation of the phospholipid analogs into the membrane and 2) the translocation assays are fast in comparison to the transbilayer movement of the analogs.

Here we have provided quantitative kinetic data on the incorporation of SL-PC into membranes as well as on assaying its transbilayer distribution, employing a stopped-flow EPR accessory with a dead time of 10 ms. Although this equipment suffers from the drawback of lacking a thermostat facility, it allows us to obtain detailed information on the limit of the spin-labeling approach outlined above. For the present purpose, in addition to liposomes, we have used erythrocyte ghosts as a model for biological membranes. By employing ghosts, we could rule out any loss of EPR intensity due to reduction of the NO moiety of SL-PC by endogenous redox reactions, which would complicate the assessment of transbilayer distribution of analogs. For example, it has been shown that spin-labeled lipid analogs with the NO moiety close to the headgroup are rapidly reduced on the cytoplasmic, but not on the exoplasmic leaflet of intact erythrocytes at 37°C (Seigneuret et al., 1984). Presumably, cytosolic redox systems such as glutathione were responsible, because no reduction of the NO moiety was observed in erythrocyte ghosts (Zachowski et al., 1986). We note that under certain conditions intracellular reduction can be employed to study the transbilayer movement of those spin-labeled lipid analogs (Seigneuret et al., 1984; Bitbol and Devaux, 1988; Müller et al., 1994).

First, we have determined the kinetics of the insertion of the SL-PC into EPC and into red cell ghost membranes. In both cases the kinetics of analog incorporation could not be fit by a single exponential. At least a biexponential function was required to provide a reasonable fit of the kinetics (see Fig. 3). Ninety percent of the SL-PC molecules became incorporated into EPC and red cell ghost membranes within 5 s at room temperature. The half-time reported for the fastest transbilayer distribution known is 20 s (see above). Therefore we conclude that incorporation of spin-labeled phospholipid analogs like SL-PC does not limit the time resolution of assays to measurement of rapid transbilayer movement. Because the kinetics of incorporation of SL-PC into EPC and into ghost membranes are very similar, we surmise that the specific composition of membranes, in particular membrane proteins, does not significantly influence the rate of the repartition process. However, this must be carefully proved for each membrane system under study. Elucidation of the mechanism of SL-PC repartition into membranes was beyond the scope of this study. Further detailed studies are warranted to investigate the transfer of the spin-labeled phospholipid analog from aqueous suspension to membranes. Several mechanisms that do not exclude each other must be considered, e.g., incorporation of analog as a monomer via the aqueous phase, and/or collision and subsequent fusion of micelles with the respective membranes.

Next we employed stopped-flow EPR spectroscopy to elucidate the time resolution that can be achieved with the ascorbate and back-exchange assays. Reduction of SL-PC in the outer leaflet of EPC vesicles by 50 mM ascorbate was

completed within ~60 s at room temperature. The reduction reaction was pseudo-first-order with respect to SL-PC. The half-time $t_{1/2}$ was ~10 s. Of course, $t_{1/2}$ was dependent on the ascorbate concentration, as demonstrated by its fivefold decrease upon an increase in the ascorbate concentration to 250 mM (data not shown). However, considering the native environment of biological membranes (ionic strength, osmotic pressure, etc.), we regard an ascorbate concentration of 50 mM as an upper limit. Ascorbate-mediated reduction of SL-PC that had been incorporated into red cell ghost membranes also followed pseudo-first-order kinetics. However, $t_{1/2}$ of the reduction at comparable conditions was slower than that obtained in the case of EPC vesicle membranes ($t_{1/2}$ = 14.5 s). Presumably, the rate of the reduction process is affected by the membrane composition, e.g., by the presence of negatively charged groups on the membrane surface. For example, anionic residues of the glycocalyx (such as sialic acids), as well as negatively charged phospholipids (like phosphatidylserine), may lower the effective concentration of the negatively charged ascorbate ions on the membrane surface by electrostatic repulsion (Schreier-Mucillo et al., 1976; Auland et al., 1994). From these results we surmise that the time resolution provided by ascorbate reduction of spin-labeled phospholipids is on the order of the half-time of the rapid transbilayer movements mentioned above and, thus, may cause serious limitations for assaying transbilayer distribution.

One way to improve the time resolution of the ascorbate assay is to replace ascorbate by a more potent reducing agent. We found that when SL-PC that resides in the outer leaflet of EPC vesicles is reduced by the ascorbic acid derivative 6-*O*-phenyl-ascorbic acid at room temperature, the reduction rate increases significantly in comparison to that of ascorbate. The calculated first-order rate constants k_r for the reduction by ascorbate and 6-*O*-phenyl-ascorbic acid differ by ~30-fold (see Table 1). This may be due to the fact that 6-*O*-phenyl-ascorbic acid is less polar than ascorbate and may therefore partly partition into the bilayer, which would raise its effective membrane concentration significantly. The latter is supported in that we found no improvement of the time resolution when using the more hydrophilic ascorbic acid derivative 6-amino-ascorbic acid in place of ascorbate (data not shown). By using symmetrically labeled EPC vesicles, we could show that ascorbate does not penetrate through EPC bilayers at room temperature. However, we found evidence for permeation of 6-*O*-phenyl ascorbic acid at room temperature. Permeation of 6-*O*-phenyl ascorbic acid across the membrane bilayer may cause a serious disadvantage, because it may hamper or even make it impossible to obtain meaningful results on the transbilayer distribution of analogs in membrane systems other than EPC vesicles. Nevertheless, the permeability of EPC membranes to 6-*O*-phenyl ascorbic acid was still sufficiently slow to allow determination of the transbilayer distribution of SL-PC in symmetrically labeled vesicles by simple extrapolation. Moreover, Wu and Hubbell (1993) have described a mathematical model that is particularly useful when the permeability of the reducing agent does not allow a simple (graphical) determination of the amount of analogs residing on the inner leaflet. The model takes into account the rate constants of reduction as well as of permeation of the reducing agent and allows an accurate determination of the transbilayer distribution. Permeation of 6-*O*-phenyl ascorbic acid did not occur when the reduction was performed at lower temperature. It could be shown that at 0°C 6-*O*-phenyl-ascorbic acid did not permeate EPC bilayers, whereas reduction of SL-PC on the outer leaflet of symmetrically labeled EPC vesicles was still very rapid (data not shown).

The transbilayer distribution of SL-PC determined for symmetrically labeled small unilamellar EPC vesicles with either ascorbate or 6-*O*-phenyl ascorbic acid was about one-third on the inner leaflet and two-thirds on the outer leaflet. This value corresponds to the theoretical distribution that can be derived mathematically for small unilamellar vesicles for which the surface area of the inner membrane leaflet is significantly smaller than that of the outer leaflet (Thomas and Poznansky, 1989).

Because the membrane spectra of SL-PC in EPC vesicles or ghosts were significantly different from the spectrum of the analog bound to BSA, we were able to measure the time course of the back-exchange of SL-PC from EPC vesicle membranes and red cell ghost membranes to BSA by means of stopped-flow EPR spectroscopy. To ensure complete label extraction, we had to use a final concentration of 5% (w/v) BSA. Lower concentrations resulted in an incomplete extraction of the SL-PC from the membrane. We note that the kinetics of SL-PC extraction could not be fitted by a single exponential. However, investigation of the underlying mechanism was beyond the scope of our study. Because the kinetics of extraction of SL-PC from the membrane of EPC vesicles and ghosts were almost identical, the presence of membrane proteins does not seem to be a major determinant of the extraction process. We found that 90% of the SL-PC molecules were extracted by 5% (w/v) BSA (final concentration) within 15 s at room temperature. Consequently, the half-time of the back-exchange assay is significantly shorter than that of the reduction assay employing ascorbate. One may therefore prefer the back-exchange approach, particularly in the case of rapid transbilayer movement. However, we must emphasize that standard protocols for this assay usually require a time-consuming centrifugation step to separate the BSA from the membranes for subsequent spectroscopic measurement of the supernatant. This step, but not the time course of analog extraction from the membrane, significantly limits the time resolution of the method. Buton et al. (1996) demonstrated that by combining the back-exchange procedure with a rapid filtration assay, a time resolution of ~30 s can be achieved. But this must be regarded as the maximum achievable time resolution. In contrast, reduction methods like the ascorbate assay do not require a centrifugation step. The time resolution is determined solely by the kinetics of the reduction reaction. The

main difficulty with the assay is controlling permeation of the reducing agent across the membrane bilayer.

We thank Mrs. S. Schiller for the synthesis of SL-PC, Mrs. B. Hillebrecht for preparing red cell ghosts, Dr. T. Pomorski for helpful comments, and Dr. A. Zachowski and Dr. P. F. Devaux for critical reading of the manuscript.

This work was supported by grants from the Deutsche Forschungsgemeinschaft to PM (Mu 1017/1-3) and AH (He 1928/2). UM is a recipient of a fellowship of the Studienstiftung des deutschen Volkes. We thank Prof. W. Lubitz for providing us with the EPR facilities within the common program Sonderforschungsbereich 312 (Deutsche Forschungsgemeinschaft).

REFERENCES

Auland, M. E., B. D. Roufogalis, P. F. Devaux, and A. Zachowski. 1994. Reconstitution of ATP-dependent aminophospholipid translocation in proteoliposomes. *Proc. Natl. Acad. Sci. USA.* 91:10938–10942.

Bitbol, M., and P. F. Devaux. 1988. Measurement of outward translocation of phospholipids across human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 85:6783–6787.

Buton, X., G. Morrot, P. Fellmann, and M. Seigneuret. 1996. Ultrafast glycero-phospholipid-selective transbilayer motion mediated by a protein in the endoplasmic reticulum membrane. *J. Biol. Chem.* 271: 6651–6657.

Calvez, J.-Y., A. Zachowski, A. Herrmann, G. Morrot, and P. F. Devaux. 1988. Asymmetric distribution of phospholipids in spectrin-poor erythrocyte vesicles. *Biochemistry.* 27:5666–5670.

Connor, J., K. Gillum, and A. J. Schroit. 1990. Maintenance of lipid asymmetry in red blood cells and ghosts: effect of divalent cations and serum albumin on the transbilayer distribution of phosphatidylserine. *Biochim. Biophys. Acta.* 1025:82–86.

Devaux, P. F. 1991. Static and dynamic asymmetry in cell membranes. *Biochemistry.* 30:1163–1173

Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100:119–130.

Dolis, D., A. I. P. M. de Kroon, and Ben de Kruijff. 1996. Transmembrane movement of phosphatidylcholine in mitochondrial outer membrane vesicles. *J. Biol. Chem.* 271:11879–11883.

Fellmann, P., A. Zachowski, and P. F. Devaux. 1994. Synthesis and use of spin-labeled lipids for studies of the transmembrane movement of phospholipids. *In* Methods in Molecular Biology. J. M. Graham and J. A. Higgins, editors. Humana Press, Totowa, NJ. 161–175.

Haest, C. W. M., G. Plasa, and B. Deuticke. 1981. Selective removal of lipids from the outer membrane layer of human erythrocytes without hemolysis. Consequences for bilayer stability and cell shape. *Biochim. Biophys. Acta.* 649:701–708.

King, M. D., and D. Marsh. 1987. Head group and chain length dependence of phospholipid self-assembly studied by spin-label electron spin resonance. *Biochemistry.* 26:1224–1231.

Klimes, N., G. Lassmann, and B. Ebert. 1980. Time-resolved electron paramagnetic resonance spectroscopy. Stopped-flow EPR apparatus for biological applications. *J. Magn. Res.* 37:3–99.

Kornberg, R. D., and H. M. McConnell. 1971. Inside-outside transitions of phospholipids in vesicle membranes. *Biochemistry.* 10:1111–1120.

Lassmann, G., L. Thelander, and A. Gräslund. 1992. EPR stopped-flow studies of the reaction of the tyrosyl radical of protein R2 from ribonucleotide reductase with hydroxyurea. *Biochem. Biophys. Res. Commun.* 188:879–887.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Martin, O. C., and R. E. Pagano. 1987. Transbilayer movement of fluorescent analogs of phosphatidylserine and phosphatidylethanolamine at the plasma membrane of cultured cells: evidence for a protein-mediated and ATP-dependent process(es). *J. Biol. Chem.* 262:5890–5898.

Menon, A. K. 1995. Flippases. *Trends Cell Biol.* 5:355–360.

Morrot, G., P. Herve, A. Zachowski, P. Fellmann, and P. F. Devaux. 1989. Aminophospholipid translocase of human erythrocytes: phospholipid substrate specificity and effect of cholesterol. *Biochemistry.* 28: 3456–3462.

Müller, K., T. Pomorski, P. Müller, A. Zachowski, and A. Herrmann. 1994. Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution of phospholipids in the plasma membrane of ram sperm cells. *Biochemistry.* 33:9968–9974.

Op den Kamp, J. A. F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* 48:47–71.

Pomorski, T., P. Müller, B. Zimmermann, K. Burger, P. F. Devaux, and A. Herrmann. 1996. Transbilayer movement of fluorescent and spin-labeled phospholipids in the plasma membrane of human fibroblasts: a quantitative approach. *J. Cell Sci.* 109:687–698.

Rousselet, A., C. Guthmann, J. Matricon, A. Bienvenue, and P. F. Devaux. 1976. Study of the transverse diffusion of spin labeled phospholipids in biological membranes. *Biochim. Biophys. Acta.* 426:357–371.

Schreier-Muccillo, S., D. Marsh, and I. C. P. Smith. 1976. Monitoring the permeability profile of lipid membranes with spin probes. *Arch. Biochem. Biophys.* 172:1–11.

Seigneuret, M., and P. F. Devaux. 1984. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc. Natl. Acad. Sci. USA.* 81:3751–3755.

Seigneuret, M., A. Zachowski, A. Herrmann, and P. F. Devaux. 1984. Asymmetric lipid fluidity in human erythrocyte membrane: new spin label evidence. *Biochemistry.* 23:4271–4275.

Thomas, P. D., and M. J. Poznansky. 1989. Curvature and composition-dependent lipid asymmetry in phosphatidylcholine vesicles containing phosphatidylethanolamine and gangliosides. *Biochim. Biophys. Acta.* 978:85–90.

Wimalasena, K., S. Dharmasena, and D. S. Wimalasena. 1994. Ascorbate based novel high affinity alternate reductants and competitive inhibitors of dopamine β-monooxygenase. *Biochem. Biophys. Res. Commun.* 200: 113–119.

Wu, G., and W. L. Hubbell. 1993. Phospholipid asymmetry and transmembrane diffusion in photoreceptor disc membranes. *Biochemistry.* 32: 879–888.

Zachowski, A. 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem. J.* 294:1–14.

Zachowski, A., E. Favre, C. Cribier, F. Galacteros, and P. F. Devaux. 1986. Outside-inside translocation of aminophospholipids in the human erythrocyte membrane is mediated by a specific enzyme. *Biochemistry.* 25:2585–2590.

Zachowski, A., A. Herrmann, A. Paraf, and P. F. Devaux. 1987. Phospholipid outside-inside translocation in lymphocyte plasma membranes is a protein-mediated phenomenon. *Biochim. Biophys. Acta.* 897:197–200.