Rapid Transbilayer Movement of Spin-Labeled Steroids in Human Erythrocytes and in Liposomes

Peter Müller and Andreas Herrmann
Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, D-10115 Berlin, Germany

ABSTRACT The transbilayer movement and distribution of spin-labeled analogs of the steroids androstane (SLA) and cholestane (SLC) were investigated in the human erythrocyte and in liposomes. Membranes were labeled with SLA or SLC, and the analogs in the outer leaflet were selectively reduced at 4°C using 6-O-phenylascorbic acid. As shown previously, 6-O-phenylascorbic acid reduces rapidly nitroxides exposed on the outer leaflet, but its permeation of membranes is comparatively slow and thus does not interfere with the assay. From the reduction kinetics, we infer that transbilayer movement of SLA in erythrocytes is rapid at 4°C with a half-time of ~4.3 min and that the probe distributes almost symmetrically between both halves of the plasma membrane. We have no indication that a protein-mediated transport is involved in the rapid transbilayer movement of SLA because 1) pretreatment of erythrocytes with *N*-ethyl maleimide affected neither flip-flop nor transbilayer distribution of SLA and 2) flip-flop of SLA was also rapid in pure lipid membranes. The transbilayer dynamics of SLC in erythrocyte membranes could not be resolved by our assay. Thus, the rate of SLC flip-flop must be on the order of, or even faster than, that of probe reduction rate on the exoplasmic leaflet (half-time ~0.5 min). The results are discussed with regard to the transbilayer dynamics of cholesterol.

INTRODUCTION

The transbilayer distribution and movement of cholesterol and of other biologically relevant steroids in membranes, in particular of cholesterol, has been the subject of numerous studies (for reviews see Schroeder, 1984; Schroeder and Nemecz, 1990; Schroeder et al., 1996). Both lipid vesicles and human red blood cells have been employed for those studies (for reviews see Schroeder, 1984; Schroeder and Nemecz, 1990; Schroeder et al., 1996). Although the phospholipid asymmetry in red blood cells is unambiguously established, there are conflicting results with respect to that of cholesterol. From investigations involving freeze-fracture electron microscopy, an enrichment of cholesterol on the exoplasmic leaflet was reported by Fisher (1976). Lange (1984), by measuring shape changes of human red blood cells, concluded that there was a symmetrical distribution of cholesterol between both leaflets (Blau and Bittmann, 1978).

In contrast, accessing cholesterol transbilayer distribution by cholesterol oxidase and by exchange proteins, a preferential orientation of cholesterol to the cytoplasmic leaflet was found by Brasaemle et al. (1988) and by Schroeder et al. (1991). Similarly, data on the flip-flop rates of cholesterol in the red blood cell membrane are contradictory; e.g., the rate of transbilayer movement of cholesterol determined by cholesterol oxidase varied between 10 s (Lange et al., 1981) and 50 min (Lange et al., 1977). However, it has been suggested that enzymatic detection of transbilayer dynamics by cholesterol oxidase perturbs the membrane and may enhance sterol transbilayer movement (Thurnhofer et al., 1986; Brasaemle et al., 1988; Schroeder and Nemecz, 1990). Other limitations of assays could be related to their time resolution. If this resolution is on the order of or above the characteristic time of transbilayer movement of the steroid molecule (or analog), both the transbilayer distribution as well as movement cannot be determined accurately.

Fluorescent analogs of cholesterol either possessing an intrinsic fluorophore such as dehydroergosterol (Fischer et al., 1985; Schroeder, 1981; Schroeder and Nemecz, 1989; Hapala et al., 1994; Schroeder et al., 1991; Mukherjee et al., 1998) or with a covalently attached reporter group such as an NBD moiety (Chattopadhyay and London, 1987; Mukherjee and Chattopadhyay, 1996; Schroeder et al., 1991; BoeszeBattaglia et al., 1996) have been used also to examine the organization of biologically significant steroids in membranes (for review see Schroeder and Nemecz, 1990). For example, using those analogs the transbilayer cholesterol distribution of human erythrocytes has been studied by two independent assays: 1) leaflet-selective fluorescence quenching of dehydroergosterol by the reactive reagent trinitrobenzenesulfonic acid and 2) measurement of lateral diffusion of NBD-labeled cholesterol by fluorescence photobleaching, which is different for the exo- and cytoplasmic leaflet (Schroeder et al., 1991). Those assays revealed an asymmetric distribution of cholesterol across the plasma membrane with ~75% of the probe present in the cytoplasmic leaflet and a rapid flip-flop between both leaflets even at low temperature. For dehydroergosterol a half-time of the transbilayer migration of ~6 min at 4°C was reported (Schroeder et al., 1991). It is not known whether such a fast transbilayer movement of cholesterol is protein mediated. Very recently, it has been suggested that

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Address reprint requests to Dr. Peter Müller, Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Biologie/Biophysik, Invalidenstrasse 42, D-10115 Berlin, Germany. Tel.: 49-30-2093-8829; Fax: 49-30-2093-8585; E-mail: peter.mueller.3@rz.hu-berlin.de.

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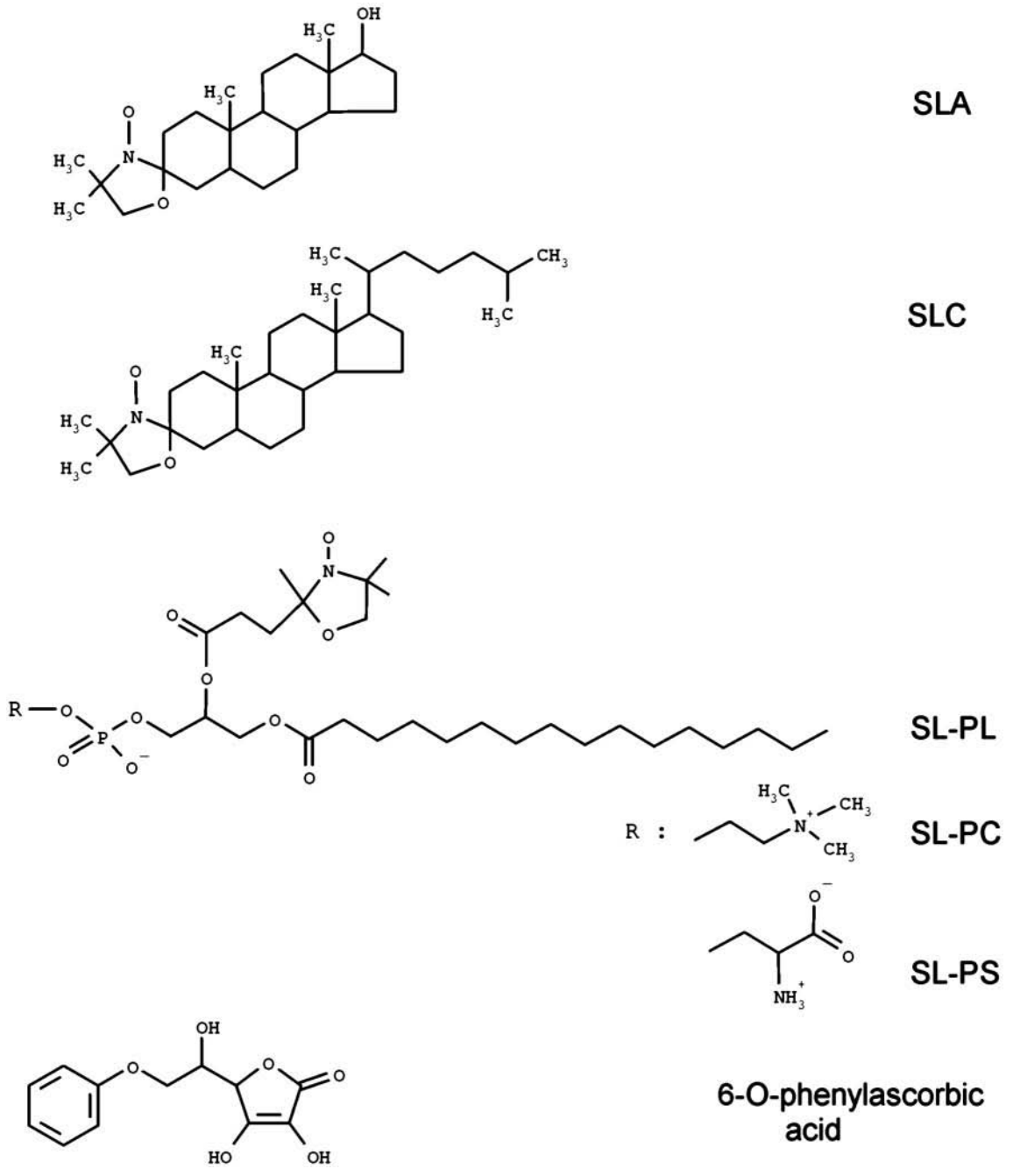

FIGURE 1 Structure of SLA, SLC, and SL-PL (respectively, of SL-PS and of SL-PC) and structure of 6-O-phenylascorbic acid.

for macrophages the ABCG1 protein is involved in transbilayer transport of cholesterol (Klucken et al., 2000).

Spin-labeled analogs of biologically relevant steroids have been widely used also to characterize the physical properties of steroids in membranes. It has been shown that the spin-labeled analogs of cholestane 3β-doxyl-5α-cholestane (SLC, Fig. 1) and androstane 3-doxyl-17β-hydroxy-5α-androstane (SLA, Fig. 1) adopt an orientation with the paramagnetic NO moiety localized in the region of the phospholipid headgroups (Morrot et al., 1987). However, unlike fluorescent analogs, they have not been successfully applied for studying the transbilayer distribution and movement of steroids so far. A major reason for that is probably a lack of appropriate assays.

In principal, the exposure of the NO moiety to the headgroup region should enable one to measure the transbilayer distribution and movement of both cholesterol analogs by leaflet specific reduction of the NO moiety into the non-

paramagnetic hydroxylamine, e.g., by ascorbate (Schreier-Mucillo et al., 1976; Grover et al., 1979). However, the accessibility of the NO moiety to ascorbic acid may be too slow to detect rapid transbilayer movement of those analogs (Morrot et al., 1987). Very recently, we have shown that the reduction of NO moieties linked to phospholipids (PLs) located in the headgroup region can be enhanced by more than one order of magnitude when using the derivative 6-O-phenylascorbic acid (Fig. 1) (Marx et al., 1997). This compound is less polar than ascorbic acid, which results in a more efficient interaction with membrane associated NO-nitroxides.

In the present study we have investigated the transbilayer movement and distribution of both molecules, SLA and SLC, in membranes of intact red blood cells as well as in liposomes by rapid reduction of the NO moiety of analogs exposed to the outer (exoplasmic) leaflet by 6-O- phenyl-ascorbic acid.

MATERIALS AND METHODS

Materials

The spin-labeled phospholipid analogs (SL-PL) of phosphatidylserine (PS), 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylserine (SL-PS), and of phosphatidylcholine (PC), 1-palmitoyl-2-(4-doxylpentanoyl)-phosphatidylcholine (SL-PC), respectively (Fig. 1), were synthesized according to Fellmann et al. (1994). 6-O-phenylascorbic acid (Fig. 1) was a generous gift of Dr. K. Wimalasena (Wichita State University, Wichita, KS). The spin-labeled analogs 3-doxyl-17β-hydroxy-5α-androstane (SLA), 3β-doxyl-5α-cholestane (SLC), the SH-reactive spin label maleimido-tempo, egg phosphatidylcholine (eggPC), and all other chemicals, if not otherwise stated, were purchased at the highest purity available from Sigma (Deisenhofen, Germany).

Preparation of erythrocytes

Citrate-stabilized blood samples of healthy donors were purchased from the local blood bank (Berlin, Germany). Red blood cells were washed once in PBS (150 mM NaCl, 5.8 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) to remove plasma and buffy coat (500 $\times$ g for 10 min at 4°C). Subsequently, two additional washes in PBS were done (2000 $\times$ g for 10 min at 4°C). In the case of SL-PS, cells before labeling with the analog were preincubated for 5 min in the presence of 5 mM diisopropyl-fluorophosphate (DFP, Aldrich, Steinheim, Germany) at 4°C to prevent hydrolysis of the analog (Calvez et al., 1988; Morrot et al., 1989).

If indicated, erythrocytes were preincubated for 15 min at 37°C with *N*-ethyl maleimide (NEM) (2 mM). After two washes in PBS, red blood cells were labeled with SLA as described below.

Labeling of red blood cells with SLA and SLC

Analogs were dissolved in chloroform at a final concentration of 1 mM (stock solution), and 36 μl of the stock solution was added to a glass tube and dried under nitrogen. In the case of SLA, the analog was dissolved in 4 μl of ethanol, and 300 μl of PBS was added. One volume of the solution was mixed with one volume of erythrocyte sediment. For SLC, the analog was dissolved by addition of 100 μl of β-methyl-cyclodextrin (5 mM) in PBS and subsequently mixed with 250 μl of red blood cell sediment. The analogs and red blood cells were incubated for 15 min at room temperature. Subsequently, red blood cell suspensions were washed three times with PBS. Red blood cells were labeled with the analogs corresponding to ~0.7 mol % of endogenous lipids.

Labeling of red blood cells with SL-PC and SL-PS and measurement of transbilayer lipid redistribution by the back-exchange assay

SL-PC and SL-PS were incorporated into the erythrocyte plasma membrane, and the amount of analog in the outer monolayer was measured using the back-exchange method as described (Calvez et al., 1988; Morrot et al., 1989). Briefly, suitable amounts of analog (corresponding to 1 mol % of endogenous phospholipids) in chloroform/methanol (1:1) were dried under nitrogen and resuspended in 1 vol of PBS. Subsequently, 2 vol of erythrocyte suspension (final hematocrit 33%) preincubated with DFP (see above) were mixed with the analog dispersion and incubated at the experimental temperature (4°C). As deduced from stopped-flow experiments incorporation of the analog into the outer leaflet occurred within 10 s (Marx et al., 1997). To assess transbilayer redistribution, at regular intervals, 80 μl of the suspension was drawn and mixed with 25 μl of ice-cold PBS containing 10% fatty-acid-free BSA. After 1 min of incubation on ice, the suspension was centrifuged (13,000 $\times$ g for 2 min), and 50 μl of the supernatant was drawn and mixed with 5 μl of 100 mM potassium ferricyanide to reoxidize reduced lipid analogs. The amount of probe present in the supernatant corresponding to lipid analogs in the outer leaflet was determined from the intensity of the EPR spectrum measured at a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany). Hydrolysis of the spin-labeled analogs into lyso derivatives that would become obvious from the appearance of three narrow peaks superimposed on the anisotropic spectrum of BSA-bound spin labels was not observed.

Preparation and labeling of liposomes

Lipids of erythrocyte plasma membrane were isolated according to the procedure of Bligh and Dyer (1959). Total phospholipid content was quantified by measuring the phospholipid phosphorus upon destruction of a lipid aliquot with 70% perchloric acid for 30 min at 180°C (Rouser et al., 1966). Cholesterol content was measured by using the Boehringer kit (Mannheim, Germany). Lipids from erythrocyte membranes contained phosphatidylcholine (35%), sphingomyelin (25%), phosphatidylethanolamine (35%), and phosphatidylserine (5%) with a molar ratio of cholesterol to phospholipid of 0.89. Erythrocyte lipids or lipids of the desired composition dissolved in chloroform:methanol (1:1 v/v) were mixed with spin-labeled analogs (1.5 mol %) and dried under a stream of nitrogen. Small unilamellar vesicles (SUVs) were prepared by sonification in PBS using a Branson sonifier W250 (Carouge-Geneve, Switzerland) at an output control setting of 2 and a duty cycle of 50% for 10 min in a glass tube cooled in an ice bath. For preparation of large unilamellar vesicles (LUVs), the dried lipid film containing spin-labeled analogs was hydrated by vortexing with PBS to give a final lipid concentration of 1 mM. The resulting aqueous phospholipid dispersion was subjected to five freeze-thaw cycles and was then extruded 10 times through two 0.1-μm polycarbonate filters (extruder from Lipex Biomembranes, Vancouver, Canada; filters from Costar, Nucleopore, Tübingen, Germany). In the case of SL-PL, this procedure gives liposomes with a symmetrical transbilayer distribution of analogs. To prepare vesicles with SL-PL exclusively located on the outer leaflet (asymmetrically labeled vesicles) liposomes were labeled after preparation.

Reduction of spin-labeled lipids by 6-O-phenylascorbic acid

The stock solution of 6-O-phenylascorbic (100 mM in PBS) was always prepared immediately before experiments. The pH of the stock solution

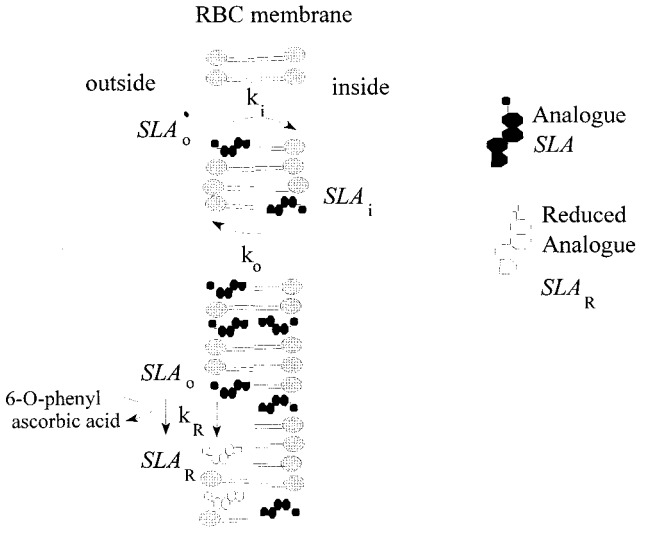

FIGURE 2 Model used for fitting the kinetics of 6-O-phenylascorbic acid reduction of spin-labeled steroids SLA and SLC in red blood cell (RBC) membranes. SLA_o and SLA_i are the amount of analogs on the outer (exoplasmic) and inner leaflet, respectively. k_i and k_o are the rate constant of the flip (inward) and flop (outward) movement, respectively. SLA_R corresponds to the analog reduced by 6-O-phenylascorbic acid. The rate constant of reduction is k_R. For details see Model and Results sections.

was adjusted to a pH of 6.8. Note that 6-O-phenylascorbic is unstable at basic pH. A 50-μl volume of precooled (4°C) labeled red blood cells (sediment) or liposome suspension was mixed with 10 μl of ice-cold stock solution of 6-O-phenylascorbic and filled into a 50-μl capillary tube (Corning, Pyrex, disposable micropipettes, Aldrich, Milwaukee, WI). Immediately after sealing, the capillary was mounted in an EPR spectrometer (ECS 106, Bruker, Karlsruhe, Germany). The temperature of the sample was adjusted by a stream of nitrogen surrounding the capillary tube. To follow the reduction of the NO moiety of analogs by 6-O-phenylascorbic acid we measured the intensity of the low-field line at 4°C as a function of time (microwave power 20 mW, modulation amplitude 4G, and magnetic field modulation 100 kHz). We did not use the mid-field line to avoid interference with the signal arising from 6-O-phenylascorbic. The earliest time point could be taken 75 s after mixing labeled membranes with the reducing substance.

MODEL

From the reduction kinetics of EPR intensity by 6-O-phenylascorbic acid, the transbilayer distribution and movement of steroid analogs in red blood cell membranes were calculated by nonlinear regression. For that purpose, we have fitted the kinetics according to the model shown in Fig. 2 (example described for SLA).

Movement of spin-labeled steroid analogs from the outer (exoplasmic) to the inner (cytoplasmic) leaflet and vice versa is characterized by the rate constants k_i (flip) and k_0 (flop). Both rate constants are assumed to be similar with $k = k_i = k_o$. Permeation of 6-O-phenylascorbic acid across membranes is slow with respect to k (see Results). Thus, in the time course of the experiment spin-label reduction by 6-O-phenylascorbic acid occurs only on the outer leaflet. The concentration of 6-O-phenylascorbic acid is in large excess over the concentration of analogs and may therefore be taken to be constant. Thus, the reduction process is pseudo-first-order with respect to analogs, and the reduction kinetics can be well described by a single exponential with k_R ($k_R = k'_R \times$ [6-O-phenylascorbic acid]). An additional precondition of the approach is that $k \ll k_R$.

SLA_o, SLA_i, and SLA_R are the intact androstane labels on the outer and inner leaflet and the reduced label, respectively. The differential equations and their analytical solution describing the model shown in Fig. 2 are as follows (the same equations are valid for the cholestane label SLC):

$$\frac{d\,SLA_o(t)}{dt} = k\,(SLA_i(t) - SLA_o(t)) - k_R\,SLA_o(t) \quad (1)$$

$$\frac{d\,SLA_i(t)}{dt} = k\,(SLA_o(t) - SLA_i(t)) \quad (2)$$

$$\frac{d\,SLA_R(t)}{dt} = k_R\,SLA_o(t) \quad (3)$$

The analytical solution is given in Marx et al. (2000). Curves were fitted to $SLA_{tot}(t) = SLA_o(t) + SLA_i(t)$ where $SLA_{tot}(t)$ respresents the label intensity at the time of ascorbate addition. Nonlinear regression was performed by using the software package Sigma Plot 4.0 (Jandel Scientific, Erkrath, Germany).

RESULTS

Reduction of spin-labeled phospholipids in erythrocyte membranes by 6-O-phenylascorbic acid

Different preconditions have to be fulfilled to access the transbilayer motion and distribution of spin-labeled lipids in red blood cell membranes by the chemical reduction of the NO moiety using 6-O-phenylascorbic acid. First, permeation of 6-O-phenylascorbic acid to the cytoplasmic leaflet and intracellular lumen of red blood cells has to be negligible in the time course of the assay. Second, reduction of analogs present in the exoplasmic leaflet has to be rapid in comparison with their transbilayer movement. To verify that hypothesis, we have probed whether those preconditions are fulfilled by using short-chain spin-labeled phospholipid analogs (for the structure of which see Fig. 1) because the transbilayer motion and distribution of those analogs are well known from former studies (Seigneuret and Devaux, 1984; Morrot et al., 1989).

Immediately after labeling of red blood cells at 4°C, the spin-labeled phospholipid analogs of phosphatidylserine (SL-PS) and of phosphatidylcholine (SL-PC) are exclusively located in the exoplasmic leaflet as probed by the back-exchange assay (see also Morrot et al., 1989).

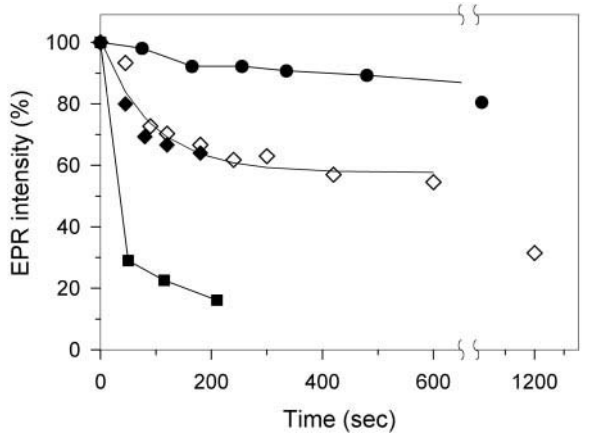

FIGURE 3 Reduction of EPR intensities of SL-PS and of 4-maleimido-tempo in red blood cells by 6-O-phenylascorbic acid. Reduction was performed in the presence of 17 mM 6-O-phenylascorbic acid at 4°C. The intensity of the low-field EPR line was measured immediately (■) or 4 h after labeling (◇ and ◆, two independent experiments) with SL-PS (incubation at 4°C) or after labeling red blood cells with 4-maleimido-tempo (●) (see Materials and Methods). Kinetics of reduction of SL- PS (◇ and ◆) were fitted to a single exponential (——).

All analogs in the exoplasmic leaflet were accessible to 6-O-phenylascorbic acid; the EPR intensity of both analogs disappeared very rapidly after addition of this reducing agent at 4°C (final concentration 17 mM; Fig. 3, SL-PC not shown). The half-time of the reduction was ~40 s. In a next set of experiments, SL-PS was allowed to redistribute to the cytoplasmic leaflet (see Materials and Methods). Inward redistribution of SL-PS is due to the action of the ATP-consuming aminophospholipid translocase (Seigneuret and Devaux, 1984; Zachowski et al., 1986). Four hours at 4°C after labeling, ~50% of SL-PS redistributed to the cytoplasmic leaflet as assessed by the back-exchange assay. Upon addition of 6-O-phenylascorbic acid to those red blood cells, we observed a rapid decline of the EPR intensity corresponding to reduction of analogs confined to the exoplasmic leaflet. After ~4 min a plateau was reached (Fig. 3).

Fitting of the data from 0 to 600 s of incubation with 6-O-phenylascorbic acid to a single exponential reveals a plateau of ~55% of analog shielded from reduction. This value corresponds to analogs located within the cytoplasmic leaflet and is in good agreement with the measurements carried out by the back-exchange assay. However, upon longer incubation in the presence of 6-O-phenylascorbic acid, we observed a further, but slow decline of the EPR intensity (Fig. 3). This is explained by a slow permeation of 6-O-phenylascorbic acid (see below) causing also reduction of analogs on the cytoplasmic leaflet after longer times of incubation. Because the passive transbilayer movement under our conditions (4°C) is very slow (Seigneuret and Devaux, 1984) we can preclude that the reduction is due to outward redistribution of SL-PS becoming exposed to 6-O-phenylascorbic acid.

We investigated the permeation of 6-O-phenylascorbic acid across the red blood cell membrane by an independent approach. For that purpose, red blood cells were incubated with the SH-reactive spin label maleimido-tempo. This label is known to permeate the red blood cell membrane and to label covalently SH groups, predominantly those of hemoglobin (Daveloose et al., 1983). After removal of unbound label, we incubated red blood cells with 17 mM 6-O-phenylascorbic acid and recorded the EPR amplitude as a function of time at 4°C. Initially, we observed a slight decrease of the EPR amplitude of ~8% (Fig. 3). Very likely, this reflects reactive groups on the membrane surface accessible to 6-O-phenylascorbic acid. However, subsequently, the signal remained almost constant. Only after incubation times above 10 min was an enhanced reduction of the intensity observed, indicating permeation of 6-O-phenylascorbic acid into the cytoplasm of red blood cells; e.g., after 17 min, ~20% of the intracellular EPR intensity disappeared. The same results were obtained when red blood cells were co-labeled with maleimido-tempo and SLA or SLC (data not shown). Although the signal of both analogs vanished rapidly upon addition of 6-O-phenylascorbic acid (see below), we found the same behavior for the EPR signal of maleimido-tempo as described for red blood cells that did not contain SLA or SLC.

However, at 14°C permeation of 6-O-phenylascorbic acid through the red blood cell membrane was rapid (curve not shown). After 10 min of incubation with 17 mM 6-O-phenylascorbic acid, the EPR intensity of maleimido-tempo decreased by ~30%. After 20 min less than 20% of the original intensity remained.

In conclusion, 6-O-phenylascorbic acid is able to reduce rapidly SL-PL localized in the exoplasmic leaflet at low temperature (4°C). Analogs on the cytoplasmic leaflet are efficiently shielded from the reduction process because permeation of 6-O-phenylascorbic acid across the red blood cell membrane was slow at low temperature. Reduction of spin-labeled groups present in the cytoplasmic leaflet was negligible during the initial 10 min. Only upon longer incubation did permeation and, thus, reduction of the latter probes become significant.

Reduction of SLA and SLC in erythrocyte membranes by 6-O-phenylascorbic acid

After labeling red blood cells with SLA (see Materials and Methods) a typical membrane spectrum (not shown) was recorded with no indication of analog clustering. Upon addition of 6-O-phenylascorbic (final concentration 17 mM) to red blood cells labeled with SLA at 4°C, we found an initial rapid reduction of the EPR intensity (Fig. 4); within 75 s it declined by ~50%. This was due

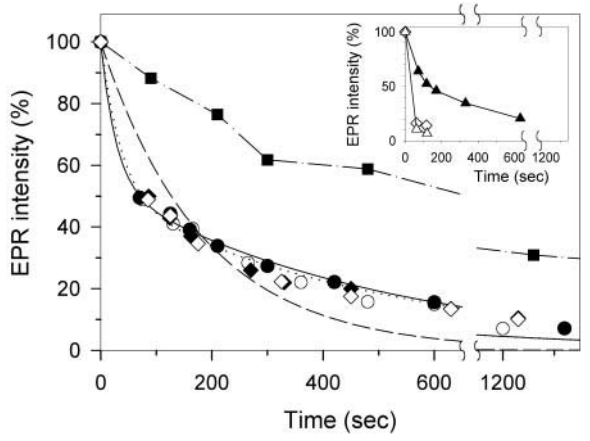

FIGURE 4 Reduction of EPR intensities of SLA and SLC, respectively, in membranes of red blood cells by 6-O-phenylascorbic or ascorbic acid. Red blood cells were labeled with SLA without (●, ■, ◇, and ◆) or with preincubation of red blood cells in the presence of NEM (○). Reduction was performed at 4°C in the presence of 17 mM ascorbic acid (■) or in the presence of 17 mM 6-O-phenylascorbic acid immediately after labeling (● and ○) or after incubating labeled cells 4 h at 25°C (◇) or at 37°C (◆). The intensity of the low-field EPR line was measured. Data for SLA (● and ○) were fitted by nonlinear regression to the model presented in Fig. 2 (——, without NEM; · · ·, with NEM preincubation). A single exponential does not fit well the data (— — —; shown only for the data of the control, without NEM preincubation). (*Inset*) Reduction of SLC at 4°C in the presence of 17 mM ascorbic acid (▲) or in the presence of 17 mM 6-O-phenylascorbic acid immediately after labeling (△) or after incubating labeled cells 4 h at 25°C (◇). Typical experiments are shown.

to reduction of SLA localized on the exoplasmic leaflet at the time of addition of 6-O-phenylascorbic acid. Subsequent to the rapid decline, a slower decrease of the intensity was observed. After 10 min only 15% of the original intensity remained. As shown above, we can preclude significant permeation of 6-O-phenylascorbic acid during this time interval. Therefore, we surmise that the slower reduction reflects redistribution of SLA from the cytoplasmic to the exoplasmic leaflet becoming accessible to 6-O-phenylascorbic acid. We note, that essentially the same reduction kinetics were measured when 6-O-phenylascorbic acid was added after labeling red blood cells with SLA for 4 h at 4°C, 25°C, or 37°C (Fig. 4, shown for 25°C and 37°C).

We have fitted the kinetics to a single exponential as well as to the model equations described above (Fig. 2) by nonlinear regression. Typical fits are shown in Fig. 4 (solid line, model; dashed line, single exponential). As is obvious, a single exponential does not fit the data at all. On the other hand, fits according to our model lead to reasonably good agreement with the experimental data. In Table 1 results of fitting kinetics from four independent experiments are summarized. The results strongly suggest that SLA is almost symmetrically distributed between both membrane leaflets. Transbilayer motion is fast with a half-time of ~4.4 min. In comparison, the half-time of SLA reduction by 6-O-phenylascorbic acid on the exoplasmic leaflet is about one order of magnitude faster (~0.5 min). Reduction was much slower when using ascorbic acid at the same concentration (Fig. 4).

Preincubation of red blood cells with NEM did not significantly affect the reduction of SLA by 6-O-phenylascorbic (Fig. 4). We obtained essentially the same values for the steady-state transbilayer distribution (58.5% on the exoplasmic leaflet) and the half-times of transbilayer motion (5.2 min) and of reduction of the NO moiety (0.46 min) from fitting of the respective kinetics to the model (Fig. 4, dotted line). After incubation of red blood cells with NEM under the same conditions, the initial rate of PS inward redistribution mediated by the aminophospholipid translocase was reduced by a factor of 50 (see also Morrot et al., 1989).

Because the cholestane analog SLC cannot be incorporated into red blood cell membranes by simple incubation of cells with the spin-labeled analog, we successfully employed the analog intercalated into β-methyl-cyclodextrin (see Materials and Methods). By incubation of red blood cells with this complex, we could incorporate SLC into the membranes. To prevent an extraction of endogenous cholesterol during the labeling procedure in the presence of β-methyl-cyclodextrin, we used a rather low concentration of β-methyl-cyclodextrin as well as a low incubation temperature (5 mM for 15 min at room temperature). Controls indicated that there were no significant changes of the cholesterol concentration in red blood cell membranes before and after incubation with β-methyl-cyclodextrin. The amount of cholesterol was 4.4 μmol/ml packed cells and 4.5 μmol/ml packed cells (average of two independent measurements) with and without incubation with β-methyl-cyclodextrin.

The reduction of SLC incorporated into red blood cells by 6-O-phenylascorbic acid was much faster in comparison to

TABLE 1 Reduction of SLA in red blood cell membranes by 6-O-phenylascorbic acid

Steady-state transbilayer distribution of SLA; SLA on the exoplasmic leaflet (%)	Flip-flop of SLA		Reduction of SLA by 6-O-phenylascorbic acid	
	k (1/min)	$t_{1/2}$ (min)	k_R (1/min)	$t^R_{1/2}$ (min)
56.6 ± 5.6	0.158 ± 0.011	4.37	1.260 ± 0.332	0.55

The EPR intensity of SLA was recorded in the presence of 17 mM 6-O-phenylascorbic acid at 4°C (typical example is given in Fig. 4). Kinetics were fitted to the model presented in Fig. 2 by nonlinear regression. Results of four independent measurements are presented as mean ± SE of estimate.

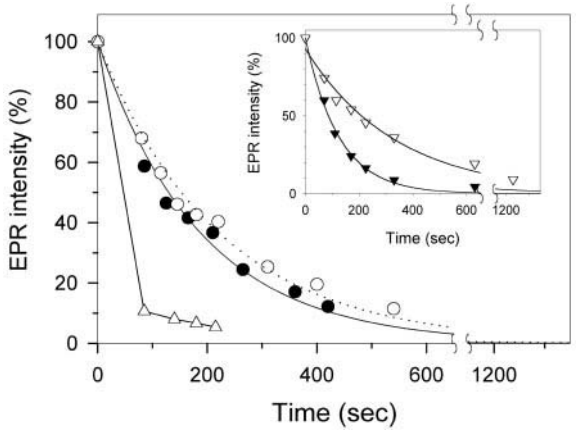

FIGURE 5 Reduction of EPR intensities of SLA (● and ○) and SLC (△), respectively, in liposomes of erythrocyte lipids by 6-O-phenylascorbic acid (17 mM) at 4°C. The intensity of the low-field EPR line was measured. Liposomes were prepared in the presence of spin-labeled analogs. Data are fitted to a single exponential (*lines*). ● and ——, SLA, LUVs; ○ and · · ·, SLA, SUVs. (*Inset*) Reduction of SLA by 6-O-phenylascorbic acid in liposomes of a phospholipid composition similar to that of the human erythrocyte membrane (PC, 30%; SM, 30%; PE, 30%; PS, 10%): ▼, without cholesterol; ▽, with cholesterol (molar ratio of cholesterol to phospholipid was 0.8). Data are fitted to a single exponential (*lines*).

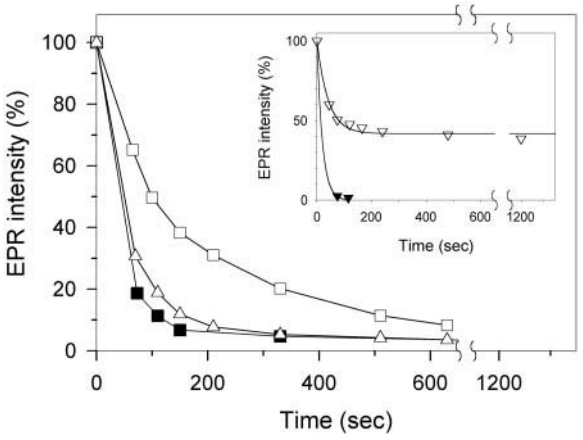

FIGURE 6 Reduction of EPR intensities of SLA and SL-PC (*inset*), respectively, in SUVs of eggPC by 6-O-phenylascorbic acid (17 mM) at 4°C. The intensity of the low-field EPR line was measured. ■, SLA in eggPC vesicles; △, SLA in eggPC:cholesterol vesicles (95:5); □, SLA in eggPC/cholesterol vesicles (2:1). (*Inset*) ▽, SL-PC, symmetrically labeled eggPC vesicles; ▼, SL-PC asymmetrically labeled eggPC vesicles.

that of SLA (Fig. 4, inset). The kinetics could be well fitted to a single exponential function. We surmise a rapid flip-flop of SLC in red blood cell membranes with a rate constant which is in the order of that of the reduction process. We preclude the hypothesis that the rapid reduction could be due to the fact that after labeling, SLC being exclusively located in the exoplasmic leaflet redistributed only slowly to the cytoplasmic leaflet. This conclusion is based on our observation that the reduction kinetics did not change when the reducing agent was added after prolonged incubation (4°C, 25°C or 37°C, 4 h) of red blood cells labeled with SLC (Fig. 4, inset, shown for 25°C). As for SLA, reduction of SLC by ascorbate was much slower in comparison to that by 6-O-phenylascorbic acid (Fig. 4, inset).

Reduction of spin-labeled lipids in liposome membranes by 6-O-phenylascorbic acid

We have measured the reduction of SLA and SLC by 6-O-phenylascorbic acid at 4°C in liposomes made from erythrocyte lipids (Fig. 5). The EPR intensity vanished completely as observed for red blood cells. As for red blood cell membranes, reduction of SLC by 6-O-phenylascorbic acid was much faster in comparison with that of SLA. The size of liposomes did not affect the reduction kinetics, because we found no difference between SUVs and LUVs (Fig. 5). However, we did not observe a biphasic disappearance of the intensity of SLA in liposomes as for red blood cells. The data can be well fitted to a single exponential (see solid and dashed line in Fig. 5). The half-times of intensity decay are 2.6 min and 2.2 min for SUVs and LUVs, respectively (for SLC 0.4 min). We surmise that the reduction of SLA or SLC by 6-O-phenylascorbic acid on the outer leaflet of liposomes is rate limiting and that the flip-flop of analogs is on the same order or even faster. Nonlinear regression showed that the model discussed above (Fig. 2) fits the kinetics too. However, a critical statistical analysis revealed that fitting was overparameterized in the latter case, the standard error of fitted parameters being on the order of their value or even larger.

Reduction of SLA by 6-O-phenylascorbic acid was studied in SUVs consisting of eggPC. We found that the EPR intensity declined more rapidly than in the case of liposomes made of lipids extracted from red blood cells (Figs. 5 and 6). Within 100 s the intensity of SLA in eggPC liposomes was reduced by more than 80%. The decay of the EPR intensity could be well fitted by a single exponential (solid line) with a half-time of 0.58 min.

Addition of cholesterol caused a slower reduction of SLA (Fig. 6). For vesicles with a molar ratio of eggPC:cholesterol of 2:1, the kinetics of reduction was very similar to that observed for liposomes of lipids extracted from erythrocytes (Fig. 5, inset). To test whether cholesterol also affects the reduction kinetics in membranes of erythrocyte lipids, we prepared liposomes containing a phospholipid composition similar to that known for the erythrocyte membrane (PC, 30%; SM, 30%; PE, 30%; PS, 10%) without and with cholesterol (molar ratio of cholesterol to phospholipid was 0.8). Reduction of SLA by 6-O-phenylascorbic acid in those liposomes became significantly slower in the presence of cholesterol (Fig. 5, inset).

Notably, we did not observe for any type of liposomes a biphasic decline of the EPR intensity upon addition of

6-O-phenylascorbic acid as found for red blood cells. Kinetics of SLA reduction in liposomes could be well described by a single exponential.

An alternative explanation for the rapid and complete reduction of SLA and SLC in liposomes could be the permeation of 6-O-phenylascorbic acid across the membrane causing also reduction of analogs present in the inner leaflet. We can rule out this explanation because we have no indication of 6-O-phenylascorbic acid permeation in the time course of the experiment. For liposomes of erythrocyte lipids labeled symmetrically with SL-PC, we observed within 200 s a reduction only of those analogs localized on the outer leaflet (not shown).

We can preclude also that 6-O-phenylascorbic acid permeates the lipid bilayer of eggPC liposomes. Upon addition of 6-O-phenylascorbic acid to SUVs labeled symmetrically with SL-PC, we observed a decrease of the EPR intensity reaching rapidly a plateau (Fig. 6, inset). As revealed from nonlinear regression (single exponential) the half-time of the decrease is 0.39 min. The plateau of ~40% corresponds to the analogs in the inner leaflet. This value is in agreement with the theoretical distribution that can be derived mathematically for SUVs for which the surface area of the inner membrane leaflet is significantly smaller than that of the outer leaflet (Thomas and Poznansky, 1989). Remarkably, the plateau remained almost stable in the time course of our experiments. This confirms our previous observation (see Marx et al., 1997) that at 4°C the permeation of 6-O-phenylascorbic acid across the liposome membrane is negligible and that 6-O-phenylascorbic acid does not trigger a flip-flop of lipids. Moreover, when SLA was simultaneously incorporated in those liposomes, we found the same stable plateau for SL-PC (data not shown; distinct parts of membrane spectra of SLA and of SL-PC could be well distinguished). Thus, the presence of SLA does not perturb the permeability properties of lipid membranes.

DISCUSSION

In the present study we have assessed the transbilayer distribution and movement of the spin-labeled steroids SLA and SLC in membranes of lipid vesicles and red blood cells by the selective reduction by 6-O-phenylascorbic acid of the nitroxides present in the exoplasmic leaflet at low temperature (4°C). We found that the transbilayer movement of SLA was rapid even at this low temperature with a half-time of ~4.3 min whereas the half-time of reduction was ~0.5 min. The half-time of SLA flip-flop is very close to that recently reported for a NBD-labeled cholesterol analog in red blood cell membranes, ~6 min at 4°C (Schroeder et al., 1991). However, we were not able to resolve the transbilayer dynamics of SLC. The half-time of SLC flip-flop was of the order or even shorter (≤0.5 min) than the characteristic time of spin-labeled steroids reduction on the exoplasmic leaflet. Nevertheless, within the experimental time course the reducing agent 6-O-phenylascorbic acid neither permeates the red blood cell membrane nor triggers a transbilayer redistribution of lipids by perturbing the membrane structure. We could not measure the fast flip-flop of SLA or of SLC by using ascorbate instead of 6-O-phenylascorbic acid. Employing the spin-labeled analog SL-PC, we have previously shown for red blood cell membranes that in comparison with 6-O-phenylascorbic acid the half-time of reduction by ascorbate is about one order of magnitude higher, even if it is used at a fivefold higher concentration (Marx et al., 1997). Thus, the rate of reduction of the NO moiety by ascorbate would be on the order of or much lower than that of the transbilayer movement of SLA and SLC, respectively. Unfortunately, our assay does not allow us to measure the transbilayer movement of steroid analogs at higher or even at physiological temperatures. Both the interference of permeation of 6-O-phenylascorbic acid as well as the expected faster flip-flop of analogs at those temperatures limit the applicability of the approach.

SLA distributes almost symmetrically between both halves of the plasma membrane. Data may indicate a slight preference for the exoplasmic leaflet (~57%) but do not support a pronounced asymmetric transbilayer arrangement. We can preclude that the assay was performed under conditions that do not correspond to the steady-state transbilayer distribution of SLA. Due to the rapid transbilayer movement, the analog adopts already during labeling of red blood cells its steady-state distribution (see Materials and Methods). Indeed, the reduction kinetics and, thus, the values of the parameters obtained from fitting were similar when the assay was performed after prolonged incubation of labeled red blood cells.

This almost symmetric distribution of SLA is in accordance with studies on human red blood cells reporting a symmetric distribution of cholesterol (Blau and Bittmann, 1978; Lange, 1984) but differs from the reported preferential localization of dehydroergosterol and of an NBD-labeled cholesterol in the cytoplasmic leaflet (Schroeder et al., 1991). For both fluorescent cholesterol analogs, it was concluded that 75% of the respective molecules are localized in this monolayer. Support for a symmetric distribution of cholesterol is given by our combined kinetic and thermodynamic approach to simulate the transbilayer asymmetry of the major lipids in the red blood cell membrane (Heinrich et al., 1997). This model takes into account the lipid composition as well as the passive and active routes of transbilayer motion of lipids in red blood cell membranes experimentally characterized in quantitative terms. Moreover, the model reflects the stationary asymmetric distribution of phospholipids under in vivo conditions provided that a rapid transbilayer movement of cholesterol of the order of minutes and a symmetric steady-state distribution of cholesterol are assumed. For a slow transbilayer movement of cholesterol, the model does not reflect the asymmetric in vivo transbilayer distribution of phospholipids at all.

The contradictory observations on transbilayer distribution may arise from the fact that due to the chemical structure the various analogs behave and distribute differently in membranes with respect to each other as well as with respect to endogenous cholesterol. Thus it remains to establish how those steroid spin labels and fluorophores reflect the behavior of cholesterol. An essential feature of cholesterol is the polar OH group, which is important for lipid interactions and the orientation of cholesterol in membranes. For membranes it is generally accepted that the OH group of cholesterol is oriented toward the lipid headgroup region and that the rigid steroid body penetrates deeply into the hydrophobic region of the lipids. Both analogs used here lack the OH group. However, the rapid reduction of the analogs in the exoplasmic leaflet of red blood cells by 6-O-phenylascorbic acid suggests that the doxyl moiety is localized in the lipid headgroup region and that the analog orientation in a lipid monolayer is similar to that of cholesterol. Indeed, it has been shown that SLC adopts only one orientation in a monolayer where the NO moiety interacts with the headgroup region but not with the methyl residues of phospholipid acyl chains (Morrot et al., 1987). Obviously, the short acyl chain of cholesterol and SLC is an important determinant of monolayer orientation. This becomes evident from the orientational behavior of SLA lacking the short acyl chain. From the observation that the NO moiety of SLA interacts with the headgroup region and with the methyl residues of phospholipid acyl chains, it is has been concluded that SLA adopts at least two orientations within a monolayer with a reorientation rate of 10^7 s^{-1} (Morrot et al., 1987). We can preclude that our assay measures the two different orientations of SLA in a monolayer (Morrot et al., 1987) rather than the transbilayer distribution of the analog. In any case reorientation of SLA must be several orders of magnitude faster than the reduction of SLA by 6-O-phenylascorbic acid in red blood cell membranes. Moreover, from the comparison of the flip-flop between SLC and SLA we surmise that the short acyl chain facilitates the transbilayer movement of steroid molecules as SLC and cholesterol.

Taken together, we conclude that due to its structure SLC resembles more closely the orientation and transbilayer movement of cholesterol in membranes. Of course, we are aware that a spin-labeled cholesterol analog preserving the typical OH group would be the best choice to characterize endogenous cholesterol. To this end, we have used such an analog bearing the NO moiety on the short acyl chain. However, this analog adopts a similar stable orientation in the membrane as cholesterol and SLC because its NO moiety was not accessible to 6-O-phenylascorbic acid. Upon addition of the reducing agent, the EPR intensity declined only very slowly (data not shown). Nevertheless, the use and comparison of the steroid analogs as SLC and SLA provide information on structural features of cholesterol determining its transbilayer movement.

A central question is whether membrane proteins are involved in transbilayer movement of cholesterol (analogs). Although we cannot preclude that an accelerated flip-flop of analogs occurs at the interface between membrane proteins and lipids, we have no indication that in red blood cells a protein-mediated transport is involved in the rapid transbilayer movement of analogs. Pretreatment of red blood cells with NEM affected neither the flip-flop nor the transbilayer distribution of SLA. It is known that NEM treatment abolishes the fast inward movement of phosphatidylserine mediated by the ATP-dependent aminophospholipid translocase (Morrot et al., 1989; Seigneuret and Devaux, 1984; Zachowski et al., 1986). Likewise, the fast, rather unspecific flip-flop of phospholipids in the endoplasmic reticulum is NEM sensitive, indicating the presence of a flippase (Herrmann et al., 1990; Buton et al., 1996; Menon et al., 2000). Moreover, if membrane proteins would be required we would expect a significantly reduced transbilayer movement of SLA or SLC in lipid vesicles. This was essentially not the case. We observed a rapid reduction of the EPR intensity of spin-labeled steroid analogs in liposomes consisting of eggPC or a phospholipid composition similar to that of red blood cell membranes. The kinetics of the intensity loss could be well described by a single exponential. Thus, we could not resolve the transbilayer distribution and movement of SLA and SLC by our assay. This strongly suggests that the reduction of the NO moiety by 6-O-phenylascorbic acid on the outer leaflet ($t_{1/2}$ of ~30 s) is the rate-limiting step rather than the transbilayer movement of the analogs. The rate-limiting role of the NO reduction process is sustained by the fact that the half-time of SL-PC reduction on the outer leaflet is quite similar to that of SLA reduction in liposomes, e.g., in eggPC vesicles, 0.4 min and 0.6 min, respectively. Thus, we cannot provide a precise value of the SLA flip-flop rate in those lipid vesicles. We can only roughly estimate that the half-time of transbilayer movement of SLA is ~30 s or even less at 4°C. Reduction of the NO moiety by 6-O-phenylascorbic acid was slower in liposomes made from red blood cell lipids but still faster in comparison with membranes of intact red blood cells. In that case, too, reduction of analogs by 6-O-phenylascorbic acid on the outer leaflet is rate limiting, and we could not distinguish between reduction of analogs on the outer leaflet and flip-flop of analogs. From the estimated rate constant of EPR intensity decline, we can conclude that the half-times of transbilayer movements of SLA and SLC are of the order of ~2.5 and 0.4 min, respectively, or even less at 4°C. Regardless of the fact that we can only give an upper limit of the half-time of analog flip-flop, the data indicate that the analogs undergo a rapid transbilayer movement even in the absence of proteins.

The rapid transbilayer movement of SLC and SLA is in agreement with the recent observation of a rapid cholesterol flip-flop in 1-stearoyl-2-oleoyl phosphatidylcholine lipo-

somes (Leventis and Silvius, 2001). The data indicated that the half-time of cholesterol flip-flop is <1–2 min at 37°C.

The difference in the reduction of analogs between the various lipid vesicles implies that the lipid composition affects the accessibility of analogs for 6-O-phenylascorbic acid and, perhaps, their transbilayer movement. We surmise that cholesterol may resemble an essential determinant of analog reduction in liposomes. As shown upon incorporation of cholesterol into eggPC liposomes or liposomes consisting of a phospholipid mixture similar to that of red blood cell membranes the reduction of SLA was significantly slower. The kinetics of reduction was quite similar to that observed for SLA in vesicles made from erythrocyte lipid extracts.

What could be the molecular basis for a rapid transbilayer movement of cholesterol (analogs)? According to its structure cholesterol is a molecule predominantly of hydrophobic nature penetrating deeply into the hydrophobic part of the membrane. Very likely, this specific nature enables a rapid transbilayer movement of cholesterol. Indeed, although phospholipids are anchored to the respective leaflet of a bilayer by their large polar headgroups preventing a rapid flip-flop in lipid bilayers, those polar elements are missing in cholesterol. It has been shown previously that cholesterol exhibits a dynamic, fluctuating motion parallel to the membrane normal, thereby protruding into the opposite monolayer of the bilayer (Gliss et al., 1999; Huster et al., 1998). We surmise that such a motion may facilitate a rapid flip-flop. Of course, apart from polar groups, the interaction with neighboring lipid components as a determinant of transbilayer movement has to be considered as well (Leventis and Silvius, 2001).

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