Phospholipid Asymmetry and Transmembrane Diffusion in Photoreceptor Disc Membranes†

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ABSTRACT: The phospholipid asymmetry and flip-flop rate in rod outer segment (ROS) disc membranes has been investigated by both a spin-label reduction method and a novel electron–electron double resonance (ELDOR) method. At 39 °C in the absence of ATP, spin-labeled derivatives of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) undergo rapid transmembrane diffusion, with a half-time of less than 10 min. At equilibrium, approximately 53% of the spin-labeled PC, 37% of the spin-labeled PE, and 18% of the spin-labeled PS reside in the inner (intradiscal) monolayer. The rapid transmembrane diffusion of phospholipids is partially inhibited by *N*-ethylmaleimide, suggesting that the process is mediated by proteins in the disc membrane.

An asymmetric distribution of phospholipids between the inner and outer monolayer is a common feature of biological membranes (Bretscher, 1972; Verkleij et al., 1973; Rothman et al., 1977; Op den Kamp, 1979; Krebs, 1982; Bishop et al., 1988; Devaux, 1991; Schroit & Zwaal, 1991). The origin, stability, and functional significance of this asymmetry are topics of current investigation in a number of systems.

The transmembrane distribution of phospholipids in photoreceptor rod outer segment (ROS)[1] disc membranes has been investigated using a variety of experimental approaches. Chemical labeling techniques have given variable results. For example, Miljanich et al. (1981), using imidoester labeling, found 77–88% of the PS and 73–87% of PE in the outer monolayer of the disc membrane. Drenthe et al. (1980a,b), employing both phospholipase hydrolysis and TNBS labeling, found little or no asymmetry in PE, PS, or PC. In contrast, Smith et al. (1977), using TNBS labeling, report that 100% of the aminophospholipids are located in the outer monolayer.

Two reports have appeared which employ nonperturbing but indirect physical approaches to infer lipid asymmetry in the disc membrane. Tsui et al. (1990) concluded that about 75% of the PS in the disc membrane must be located in the outer monolayer on the basis of the electrostatic properties of the membrane surface. Sklar and Dratz (1980) noted that the thermal properties of the disc membrane could be approximately accounted for by a model in which the inner monolayer contains most of the PC and cholesterol, while the outer monolayer is enriched in PS and PE.

Chemical labeling techniques such as those used to study lipid asymmetry in the disc membrane have been reviewed by several authors (Op den Kamp, 1979; Etemadi, 1980; Krebs, 1982). One of the critical assumptions underlying these techniques is that the lipid asymmetry is stable during the time course of the study. Since the development of these strategies, it has become appreciated that lipid transmembrane migrations (flip-flop) can be quite rapid, and it is essential to investigate the transmembrane migration kinetics of phospholipiids in the disc membrane before accepting the validity of such studies.

Devaux and co-workers have developed a spin labeling approach for determining both the kinetics and equilibrium distribution of phospholipids in membrane systems (Seigneuret et al., 1984). This technique does not require significant modification of the membrane properties and has been successfully used in measuring the asymmetric distribution of phospholipids in erythrocytes and other membrane systems (Herrmann et al., 1990; Sune et al., 1987, 1988; Zachowski et al., 1987; Seigneuret et al., 1984). This method employs phospholipids with the desired head group in which the β chain is relatively short and contains a nitroxide spin label. The water solubility of such molecules is sufficiently high that they may be readily incorporated into the outer monolayer from the external solution. The subsequent transmembrane migration is followed either by reducing the external nitroxide with an impermeable agent like ascorbate or by extracting it with bovine serum albumin (BSA) at various time points. The remaining EPR signal is due to internalized nitroxide, and the growth of this signal in time may be analyzed to give the inward translocation rate constant. In the present work, this approach was used with ascorbate reduction to determine the asymmetry and flip-flop rate of phospholipids in disc membranes.

The ascorbate reduction method requires that the reduction rate of the nitroxide in the outer monolayer be much faster than either the leakage of ascorbate across the membrane or the flip-flop rate of the lipids. This condition is not met for disc membranes at physiological temperature, and an aliquot of the sample must be cooled to near 0 °C to reduce ascorbate leakage to an acceptable level. Even at low temperature, there is a slow reduction of the inner monolayer population, and data must be collected as a function of time and extrapolated to time zero to obtain the required value for the internal

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[1] Abbreviations: ROS, rod outer segment; ELDOR, electron–electron double resonance; PC, phosphatidylcholine; PCSL, phosphatidylcholine spin label; PE, phosphatidylethanolamine; PESL, phosphatidylethanolamine spin label; PS, phosphatidylserine; PSSL, phosphatidylserine spin label; TNBS, 2,4,6-trinitrobenzenesulfonic acid; BSA, bovine serum albumin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; DFP, diisopropyl fluorophosphate; DTAB, dodecyltrimethylammonium bromide; ER, endoplasmic reticulum; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; EPR, electron paramagnetic resonance.

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population. Thus for each time point, a complete time course of reduction at low temperature must be obtained.

In this paper we introduce a new strategy based on ELDOR spectroscopy that is capable of directly observing the transbilayer distribution of spin-labeled lipids in a noninvasive and time-resolved manner.

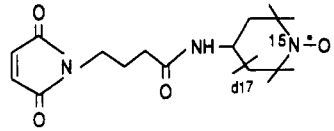
The results of the present investigation demonstrate that, at physiological temperature, the phospholipids of the disc membrane undergo rapid transmembrane flip-flop, with a half-time under 10 min, and are asymmetrically distributed across the bilayer. The asymmetry is in good agreement with that predicted by the transbilayer coupling model (Hubbell, 1990).

MATERIALS AND METHODS

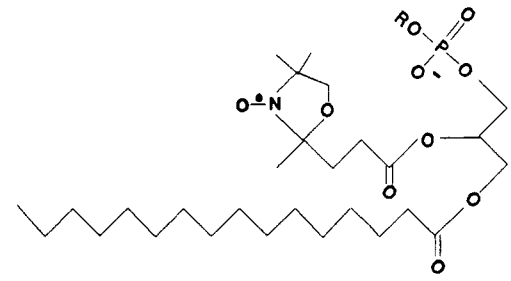
Dark-adapted frozen bovine retinas were obtained from J. A. Lawson (Lincoln, NE) and were stored at −80 °C until needed. Monopalmitoylphosphatidylcholine was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), and 4-amino-[1-^{15}N]-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} was obtained from MSD Isotopes (Montreal, Canada). The ATP assay kit was obtained from Sigma (St. Louis, MO).

HPLC was performed on a Varian 5060 with a Hewlett-Packard 1040M diode array detector. The X-band ELDOR spectrometer is based on a two-loop–one-gap resonator (Hubbell et al., 1987). It is of the same general design as that of Hyde et al. (1985) but includes a low-noise microwave preamplifier before the detector and an automatic frequency lock arm on the pump klystron. NMR spectra were obtained on a Bruker AM360 spectrometer. Mass spectra were obtained on a VG ZAB-SE spectrometer.

Synthesis of 4-(4′-Maleimidobutyramido)-[1-^{15}N]-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} (MSL). *N*-(4-Maleimidobutyryloxy)succinimide was synthesized according to the method of Rich (1975). This compound (50.4 mg, 0.18 mmol) and 4-amino-[1-^{15}N]-1-oxy-2,2,6,6-tetramethylpiperidine-d_{17} (34 mg, 0.18 mmol) were mixed in 1 mL of THF and stirred for 12 h at room temperature. The solvent was removed by rotary evaporation, 3 mL of methylene chloride was added to dissolve the residue, and the solution was washed 2 times with 3 mL of water. The organic layer was dried with anhydrous magnesium sulfate and the product was purified by flash liquid chromatography on 30–60 mesh silica. The column was presaturated with ether and eluted with chloroform/methanol/water at 65:25:4. The yellow nitroxide band was collected, giving 52 mg (yield 82%) of a yellow semisolid. The compound ran as a single spot on silica gel TLC with chloroform/methanol/water (65:25:4) as an eluant. High-resolution mass spectrometry gave a molecular ion of m/e = 354.2935 a value 2.5% lower than that expected for $C_{17}H_9D_{17}N_2{}^{15}NO_4$.



Synthesis of Spin-Labeled Phospholipids. The general structure of the spin-labeled phospholipids used in this study is shown below. The spin-labeled PC (PCSL) was synthesized according to Hubbell and McConnell (1971). The PE and PS spin labels (PESL and PSSL) were prepared enzymatically from PCSL according to Comfurius and Zwaal (1977) and Griffith et al. (1986) with a modification on the final purification steps. Both PESL and PSSL were purified by preparative HPLC on a silica gel column (normal phase Micro Pak Si-10, 300 × 8 mm). For the purification of PESL, the solvents were hexane/2-propanol/0.1 M ammonium bicarbonate at 40:58:2 (solvent A) and 40:50:10 (solvent B). The elution program was as follows: 0–5 min, 100% A; 5–35 min, 100% A to 0% A linearly; 35–90 min, 100% B. PESL elutes at 45 min and PCSL elutes at 68 min.



PCSL: R = choline
PESL: R = ethanolamine
PSSL: R = serine

For the purification of PSSL on the same column with the same flow rate, the solvents were hexane/2-propanol/water/concentrated sulfuric acid at 97:3:0:0.02 (solvent A) and 40:50:10:0.02 (solvent B). The elution program was as follows: 0–10 min, 100% A; 10–40 minute, 100% A to 0% A linearly; 40–90 min, 100% B. PSSL elutes at 40 min as a single peak. PCSL, PESL, and PSSL purified by HPLC gave a single spot on silica gel TLC with chloroform/methanol/water (65:25:4) as the eluant.

Isolation of Rod Outer Segment Membranes and ATP Assay. ROS disc membranes were isolated from 50 dark-adapted frozen bovine retinas under dim red light according to the procedures described by Schnetkamp et al. (1979). All sucrose solutions were made with a buffer containing 20 mM HEPES and 0.2 mM EDTA, pH 7.4. The ROS were collected from the uppermost dense band and washed with 20 mM MOPS buffer, pH 7.0, three times. Rhodopsin concentration was determined spectrophotometrically by the absorbance at 500 nm (A_{500nm}) after solubilization in 50 mM octyl glucoside. The ratio A_{280}/A_{500} was 2.2. The membranes were used within 3 days after isolation. All buffers were bubbled with argon before use, and membrane suspensions were kept under argon during all incubations. The membranes were preincubated with 5 mM DFP at 30 °C for 3 h and maintained in 5 mM DFP during flip-flop measurement to minimize the hydrolysis of spin-labeled phospholipid. The ATP content of the membranes was estimated with the procedure described in the ATP bioluminescent assay kit from Sigma, and the emitted light was detected spectrofluorometrically as described by Holmsen et al. (1966).

Preparation of Unilamellar Egg PC Vesicles. Egg PC was suspended in 100 mM HEPES, pH 7.4, at a concentration of 1 mg/mL and freeze–thawed five times. The resulting suspension was extruded 8 times through a 500-Å membrane filter, yielding a translucent suspension of vesicles. In another preparation, PCSL at 2% of the total lipid was mixed with egg PC at a concentration of 1 mg/mL, freeze–thawed, and extruded with 500-Å membrane as described above.

Reconstitution of Egg PC Vesicle Containing Rhodopsin. Rhodopsin was purified in DTAB by hydroxyapatite chromatography (Hong et al., 1982). Rhodopsin was further purified on a concanavalin A affinity column (Litman, 1982). The purified rhodopsin in 30 mM octyl glucoside solution gave an absorption ratio of A_{278}/A_{498} = 1.6. The purity of rhodopsin was also checked by SDS–PAGE with silver staining. Only a single rhodopsin band could be visualized.

The purified rhodopsin in octyl glucoside was mixed with egg PC with a molar ratio of 1:100, rapidly diluted, and

dialyzed to remove octyl glucoside (Jackson & Litman, 1985). The resulting vesicles were washed several times with 100 mM HEPES at pH 7.4 and concentrated in a Centricon 30 (Amicon, Beverly, MA) to a final concentration of about 0.1 mM rhodopsin. The vesicle size distribution was examined by electron microscopy after fixation with 2% osmium tetraoxide and negative staining with 1% uranyl acetate.

To obtain reconstituted vesicles with PCSL in both monolayers, egg PC containing 2% of PCSL was mixed with the purified rhodopsin solution in the above procedure. In this way, PCSL was incorporated into the vesicles uniformly and it should equally distribute between the inner and outer monolayer. These vesicles were used to check the validity of the ascorbate reduction method in reconstituted vesicles.

Flip-Flop Measurement by the Spin-Label Reduction Method. The distribution of the spin-labeled lipids in ROS membranes was determined by ascorbic acid reduction essentially according to Seigneuret and Devaux (1984). Isolated ROS membranes were resuspended in 100 mM Hepes (pH 7.4) with 5 mM DFP and the membranes were pelleted by centrifugation at 12000*g* for 20 min. Spin-labeled phospholipids PCSL, PESL, and PSSL (10 mM stock solution in 100 mM HEPES buffer, pH 7.4) were added to the membrane pellets and the suspension was incubated at 39 °C for the desired times. The spin labels did not exceed 1% of the total lipids in the ROS membranes. At different times, an aliquot of the suspension was cooled rapidly on ice to 1 °C and cold ascorbic acid in 100 mM HEPES, pH 7.4, was then added to the membranes to a final concentration of ascorbic acid of 50 mM. The reduction of the intensity of the center line of the EPR spectrum was followed with time at 1 °C. In all cases, a fraction of the spin was rapidly reduced, leaving a protected or slowly reducing EPR signal corresponding to the spin labels that reside in the inner monolayer. The percentage of spin label in the inner monolayer was obtained by extrapolation of the slow reduction curve to time zero. Population data obtained in this way as a function of time was used as discussed below to determine the derived kinetic constants and equilibrium population. The same ascorbate reduction procedure was used to measure the transmembrane distribution of PCSL in unilamellar egg PC vesicles and in egg PC reconstituted membranes containing rhodopsin.

Modification of Rhodopsin with MSL. Rhodopsin sulfhydryl groups were modified with MSL by adding the desired amount of a stock solution (100 mM in ethanol) to a suspension of ROS membranes (2 mg/mL rhodopsin in MOPS buffer, pH 7.0) and stirring in the dark at 30 °C. The molar ratio of MSL to rhodopsin was approximately 20:1 and the amount of ethanol did not exceed 1% (v/v). To determine the amount of time necessary for complete reaction, aliquots of the reaction mixture were taken out at different times and the membranes were washed three times with buffer. The amount of reacted spin label was determined by double integration of the first derivative EPR spectrum. The concentration of rhodopsin in the aliquot was determined after solubilization in 50 mM octyl glucoside by the absorption at 500 nm using an extinction coefficient of 42 600 M^{-1} cm^{-1}. The ratio of moles of spin label to moles of rhodopsin was then calculated as the ratio of the above values.

Phospholipid Flip-Flop and Distribution Measurement by ELDOR. ROS membranes with MSL-labeled rhodopsin on two cysteines were mixed with PCSL, PESL, or PSSL. The spin-labeled lipids did not exceed 1% of total lipids in ROS. The mixture was immediately transferred to a TPX gas-permeable sample capillary. Nitrogen gas was passed through the resonator around the capillary to remove oxygen and to provide temperature control at 39 °C. The EPR field modulation frequency was 100 kHz and the incident observing power was 0.4 mW.

For determination of ELDOR reduction, the ^{14}N nitroxide on the lipid was chosen as the "pumped" species, and the center line ($M_I = 0$) was irradiated with an intense microwave field. The ^{15}N nitroxide on the protein was the "observed" species, and the low-field resonance ($M_I = -1/2$) was monitored. The separation between the pumping frequency and observing frequency was 33.2 MHz [see Hyde and Feix (1989) for a general review of ELDOR spectroscopy applied to biological systems].

In the ELDOR experiment, the fractional reduction in intensity of the observed spectral line due to the pump is determined. Because of some spectral overlap between the ^{15}N and ^{14}N nitroxides at the position of the observed resonance, a correction must be made for the contribution of the ^{14}N nitroxide and its amplitude change during pumping. This was accomplished using an otherwise identical sample which contained only the ^{14}N isotope. The overlap is not severe, and the corrections amounted to no more than 10% of the amplitude changes in the composite spectrum up to 100 mW of pumping power. ELDOR reduction factors up to 50 mW were obtained as previously described (Popp & Hyde, 1982; Feix et al., 1984).

THEORY

Theory of the ELDOR Method for Determining Transmembrane Diffusion Rates and Equilibrium Asymmetry of Spin-Labeled Lipids. Rhodopsin is highly oriented in the disc membrane and contains two reactive sulfhydryl groups on the external surface of the membrane (Chen & Hubbell, 1978; Fung & Hubbell, 1978a), probably due to Cys 316 and Cys 140 (Pogozheva et al., 1985; Findlay et al., 1988). These sulfhydryls provide a convenient and stable marker for the external surface and are readily labeled with a variety of nitroxide reagents (Favre et al., 1979; Delmelle & Virmaux, 1977). In the present experiments, one or both of these sulfhydryls are labeled with the ^{15}N-containing nitroxide MSL described above.

If a suitable lipid containing an ^{14}N nitroxide, such as the phospholipid spin labels described above, is added to the external monolayer, collisions will occur between the fixed ^{15}N nitroxide on the protein and the ^{14}N nitroxide on the lipid (see Figure 1). The collision frequency of each protein-bound label with the lipid is proportional to the concentration of the lipid in the outer monolayer. As flip-flop of the lipid occurs to the inner monolayer, the collision frequency will decrease proportionally. Thus, any method that can determine the collision frequency between the label on the lipid and that on the protein can be used to directly observe and quantitate flip-flop.

When ^{15}N and ^{14}N nitroxides collide, they exchange spin states with a probability of $1/2$ (Freed, 1966). This process is called Heisenberg exchange (HE) and is directly proportional to the collision frequency and hence concentration. The relevant relationship is

$$W_{HE}^{L\rightarrow P} = p4\pi dD[L]_o \quad (1)$$

where $W_{HE}^{L\rightarrow P}$ is the HE rate experienced by a single fixed nitroxide due to the labeled lipid of concentration $[L]_o$, D is the relative diffusion constant, p is the probability of effective exchange per collision, and d is the collision diameter (Eastman et al., 1969).

The HE rate is directly measurable by ELDOR spectroscopy. The subject of dual-label $^{14}N/^{15}N$ ELDOR has been

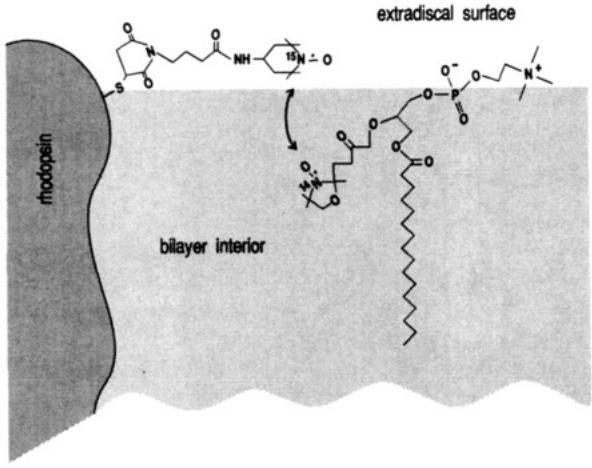


FIGURE 1: Schematic illustration of the ELDOR experiment. Cysteine residues near the extradiscal membrane–solution interface are labeled with an ^{15}N nitroxide-maleimide (MSL), in which the nitroxide group is extended away from the protein surface by a four-carbon spacer arm. An ^{14}N spin-labeled phospholipid is incorporated into disc membranes at a concentration of 1% of the native lipid in disc membranes. The collision frequency of the ^{14}N and ^{15}N nitroxides is proportional to the lipid concentration in the outer monolayer at any time.

recently reviewed (Hyde & Feix, 1989) and will only be briefly discussed here. In the dual-label ELDOR experiment, one spin population is irradiated with an intense microwave source (the "pump" source) at frequency ν_p and the response is observed at the other spin population with a weak source (the "observe" source) at frequency ν_o. HE transfers saturation from the pumped to the observed species in proportion to the rate of collision, and the effect is a reduction in the intensity of the observed species.

The ELDOR reduction factor, R, is defined as (Hyde et al., 1968)

$$R \equiv 1 - I_{on}/I_{off}$$

where I_{on} and I_{off} are the spectral intensities of the observed species with the pumping power on and off, respectively. The reduction factor is a function of pumping power as well as the HE rate. To eliminate the power dependence, a plot of $1/R$ vs 1/power is linearly extrapolated to infinite power to give the reduction factor at infinite power, R_∞. For strong coupling between the nitrogen hyperfine states, this quantity is directly related to the exchange rate according to (Yin & Hyde, 1987)

$$R_\infty^{-1} - 1 = 2W_e/W_{HE}^{L\rightarrow P} \quad (2)$$

where W_e is the electron spin–lattice relaxation rate for the nitroxide.

Combining eqs 1 and 2 and rearranging gives

$$[L]_o = W_e\rho/\pi dD \quad (3)$$

where $\rho = (R_\infty^{-1} - 1)^{-1}$. Equation 3 shows that the concentration of labeled lipid in the outer monolayer is directly proportional to the experimentally derived quantity ρ. Thus the rate of transmembrane migration can be followed by determining ρ as a function of time.

Kinetic Model of Phospholipid Transmembrane Diffusion. If $[L]_o$ and $[L]_i$ are the concentrations of spin-labeled phospholipid in the outer and inner monolayer of membranes, respectively, the first-order kinetic scheme for transport will be taken as

$$[L]_o \underset{k_i}{\overset{k_o}{\rightleftarrows}} [L]_i$$

where k_i and k_o are the first-order inward and outward rate constants, respectively.

With the condition that all of the labeled phospholipids reside in the outer monolayer at time zero, the integrated rate law gives the time dependence of the concentrations as

$$[L]_o = ([L]_{o0} - [L]_{o\infty}) \exp[-(k_o + k_i)t] + [L]_{o\infty} \quad (4)$$

$$[L]_i = \frac{k_i[L]_{o0}}{k_i + k_o}[1 - \exp[-(k_i + k_o)t]] \quad (5)$$

where $[L]_{o0}$ is the concentration of spin label in the outer monolayer at time zero (total spin label concentration) and $[L]_{o\infty}$ and $[L]_{i\infty}$ are the equilibrium concentrations of labeled phospholipid in the outer and inner monolayer, respectively.

The ELDOR method described above determines ρ, a quantity proportional to the amount of label in the outer monolayer. Thus eq 4 will be used to analyze the ELDOR data with the appropriate value of ρ replacing the [L] variable. On the other hand, the ascorbate reduction method yields directly the spectral intensity, I, of spin label on the inner monolayer. Thus eq 5 will be used to interpret data from the ascorbate reduction experiments with the appropriate spectral intensity replacing the [L] variable.

RESULTS

Characterization of Vesicles. ROS membrane vesicles prepared according to procedures similar to those used here have been previously characterized with respect to protein and lipid composition (Daemen, 1973), rhodopsin orientation within the membrane (Chen & Hubbell, 1973), and vesicle geometry (Amis et al., 1981). Of particular importance to the experiments conducted here are the purity of the preparation and preservation of the native orientation of rhodopsin in the membrane. Polyacrylamide gel electrophoresis in SDS indicated that >90% of the protein was due to rhodopsin (data not shown), characteristic of highly purified disc membranes (Daemen, 1973). Consistent with this high degree of purity was the spectral absorbance ratio, A_{280}/A_{500}, which was 2.2. Freeze-fracture electron microscopy has shown that the rhodopsin molecule retains its native orientation in the isolated vesicles (Chen & Hubbell, 1973). This can be verified chemically through the use of proteolytic cleavage. Rhodopsin was a thermolysin and trypsin cleavage site in the third cytoplasmic loop, on the external surface of the vesicles, but none on the intradiscal surface (Pober & Stryer, 1975; Fung & Hubbell, 1978a). Thus, properly oriented vesicles will result in all rhodopsin molecules being cleaved by the protease. Rhodopsin in the inverted orientation is protected from proteolysis (Fung & Hubbell, 1978b). Thermolysin proteolysis of the vesicles used in these experiments results in rapid cleavage of all rhodopsin, indicating a native orientation of the protein (data not shown).

Finally, the vesicle preparation contains less than 10^{-8} M ATP, in the absence or presence of 20 mM octyl glucoside to solubilize the membranes. This is important, since it establishes that the rapid lipid movements detected are not driven by ATP hydrolysis.

Transmembrane Diffusion and Equilibrium Asymmetry Determined by the ELDOR Method. Initial experiments revealed that the spin-labeled phospholipids are rapidly hydrolyzed at the *sn*-2 position in ROS membranes, as demonstrated by the appearance of a sharp EPR spectrum corresponding to a free spin-labeled fatty acid in the aqueous phase. No detectable hydrolysis was observed in pure lipid bilayers of egg phosphatidylcholine. A similar rapid hydrolysis

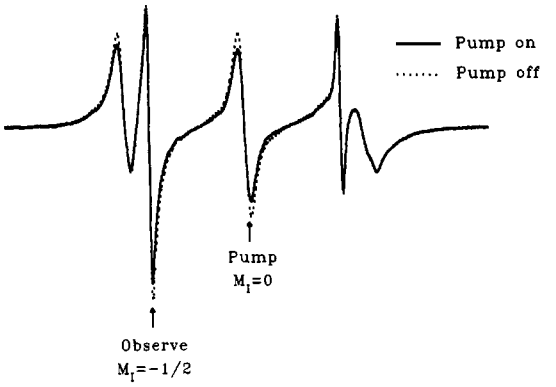


FIGURE 2: Composite EPR spectrum of MSL-labeled rhodopsin and PSSL in ROS membranes with and without pump power. The ^{14}N nitroxide center line $m_I = 0$ is pumped and the ^{15}N nitroxide $m_I = -1/2$ line is observed for ELDOR reduction. The frequency difference between observing and pumping is 33.2 MHz. The spectrum was taken at 39 °C.

of spin-labeled phospholipids in native membranes has been previously reported by Bitbol and Devaux (1988) and Lai et al. (1988). At 39 °C, almost all spin-labeled phospholipids in ROS membranes were hydrolyzed within 6 h. This hydrolysis makes the ELDOR experiment as outlined above impossible. Since diffusion of the water-soluble nitroxide fatty acid is much faster than that of the phospholipid confined to the bilayer, even a relatively small amount of hydrolysis causes problems due to the resulting increase in collision frequency between the ^{15}N and ^{14}N nitroxides.

The hydrolysis could not be inhibited by either 2,4-dibromoacetophenone or Zn^{2+}, well-known inhibitors of phospholipase A_2. However, the presence of 5 mM DFP during the measurement period reduced the amount of hydrolysis to no more than 5% during a 3-h measurement period [see Bishop and Bell (1985) and Bitbol and Devaux (1988) for the use of DFP]. Thus, all data presented below were obtained in the presence of 5 mM DFP.

At 30 °C, pH 7.0, 2.3 ± 0.2 sulfhydryl groups are modified per rhodopsin in the presence of 1 mM MSL. The reaction is complete at about 5 h in the dark. Figure 2 shows an EPR spectrum of rhodospin labeled at both sites with ^{15}N-MSL in the presence of PSSL in ROS membranes at 39 °C. This temperature was selected since it is close to the physiological temperature for the bovine ROS membranes (38.6 °C) and yields sharper spectral features than at room temperature. The dotted line in Figure 2 is the spectrum in the absence of pump power, and the solid line corresponds to an incident pumping power of 15 mW. The ELDOR reduction factor at the negative branch of the $M_I = -1/2$ signal is determined as a function of pumping power, and the data are plotted as shown in Figure 3. The intercept on the ordinate gives R_∞^{-1} and hence $\rho = (R_\infty^{-1} - 1)^{-1}$, which is directly proportional to the concentration of lipid in the outer leaflet of the disc membrane at any time (eq 3).

To quantitatively interpret the ELDOR data, the relative efficiencies of exchange of the two nitroxides on rhodopsin with the spin-labeled lipid must be determined. To this end, the differential reactivity of the two sulfhydryl groups of rhodopsin in the disc membrane at pH 7.0 (Chen & Hubbell, 1978) were used to prepare samples with the MSL label amounting to greater than 80% of the total label at either rhodopsin site (Wu, 1992). ELDOR experiments of the type shown in Figure 3 were carried out on such selectively labeled samples, and the results were indistinguishable from those with both sites labeled. This indicates that MSL at each sulfhydryl group is equivalent with respect to exchange with

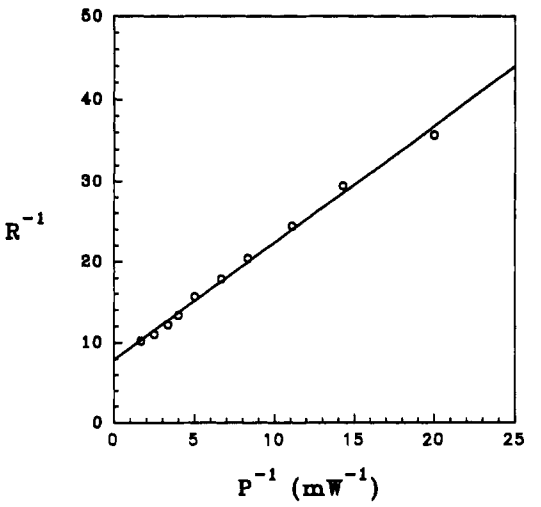


FIGURE 3: Plot of the inverse of the ELDOR reduction factor, R, versus the inverse of the microwave pumping power. The inverse reduction factor at infinite power, R_∞^{-1}, is obtained by linear extrapolation.

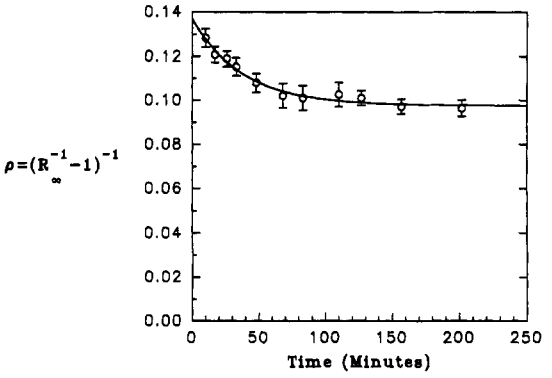


FIGURE 4: Flip-flop rate and transmembrane distribution of PSSL in disc membranes as measured by ELDOR. The solid line is obtained by fitting the data to eq 4. The error bars were determined from the uncertainty in the linear extrapolation of the R^{-1} versus P^{-1} plot to determine each R_∞^{-1}. The experiment was done at 39 °C in 20 mM MOPS, pH = 7.0.

the labeled lipid, which is consistent with recent structural models of rhodopsin (Farahbakhsh et al., 1992). Therefore, the labeled cysteines are indistinguishable and may be treated with regard to exchange as a single homogeneous population according to eqs 1–3 above.

Figure 4 shows a plot of ρ as function of time for PSSL in disc membranes. The solid curve is a least-squares fit of the data to eq 4. The fit gives values of $\rho(t = 0) = 0.137 \pm 0.004$, $\rho(t = \infty) = 0.098 \pm 0.001$, and $k_o + k_i = 0.027 \pm 0.004$ min^{-1}. The percentage of PSSL in the outer monolayer at equilibrium is $\rho(t = \infty)/\rho(t = 0) = 72\% \pm 3\%$. The transmembrane migration process is first-order with a half-time of 26 ± 4 min.

When similar experiments were carried out with PCSL and PESL, ρ was time-independent over a period of 3 h. Thus there is little transmembrane migration of these lipids during a period that allows PSSL to reach equilibrium. This unusual result will be discussed below in light of the ascorbate reduction experiments.

Transmembrane Diffusion and Equilibrium Asymmetry of Phospholipid Determined by the Ascorbate Reduction Method. The ascorbate reduction method for determination of the instantaneous transmembrane distribution of spin-labeled phospholipids requires that the sample be rapidly cooled to reduce the transmembrane diffusion rates of both the ascorbate and spin label. In the present experiments, the

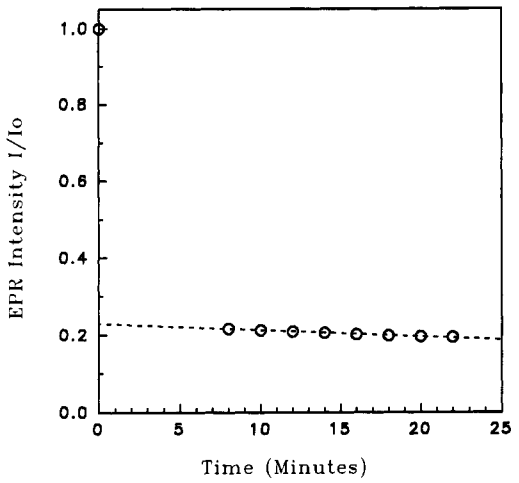


FIGURE 5: Reduction of PESL in ROS membranes by 50 mM ascorbic acid at 1 °C, pH 7.4, buffered with 100 mM HEPES. An initial rapid reduction followed by a slower reduction of the EPR signal is apparent.

membranes were cooled to 1 °C. The EPR spectrum of spin-labeled phospholipids in ROS membranes at this temperature is broad with a superimposed low-amplitude sharp peak, due to free fatty acid from the hydrolytic reaction mentioned above. Subtraction of the spectrum of free spin-labeled fatty acid in water from the composite spectrum followed by double integration showed that the water-soluble spin label constituted less than 2% of the total at the beginning of the experiment. After 3 h at 39 °C in the presence of 5 mM of DFP, this water-soluble spin label increased to no more than 7%.

A typical spin-label reduction profile by ascorbic acid is shown in Figure 5. The spin-labeled lipid residing in the outer monolayer is rapidly reduced, leaving a protected population corresponding to spin-labeled lipid in the inner monolayer of the disc membranes. The protected population is very slowly reduced, due either to ascorbic acid entry into the disc interior or to the slow outward transmembrane movement of phospholipid at low temperature. The high activation energy reported for phospholipid flip-flop (Kornberg & McConnell, 1971) would make this process much slower than the residual reduction at 1 °C and pointed to the leakage of ascorbate as the dominant process. The reduction rate is sufficiently slow that a linear extrapolation to zero time may be used to obtain the fraction of spin-labeled phospholipid residing in the inner monolayer of disc membranes at the time of ascorbate addition (see Appendix). The fraction of internalized lipid determined in this way as a function of time for the three spin-labeled lipids is shown in Figure 6 for three independent experiments on different membrane preparations. The parameters obtained from least-squares fitting of the data to eq 5 are summarized in Table I. The transmembrane diffusion of all the three spin-labeled phospholipids is relatively rapid, with half-times under 10 min.

The ELDOR approach gave 28% of PSSL in the inner monolayer at equilibrium, compared to 18% for the reduction method. In addition, the rate constant for approach to equilibrium for this lipid is about a factor of 2 slower when determined by the ELDOR approach as compared to the reduction method, and neither PCSL nor PESL underwent transmembrane migration as determined by ELDOR. The source of these interesting differences appears to be the treatment of the disc membrane with a sulfhydryl reagent (MSL spin label) in the ELDOR method.

To investigate the effect of sulfhydryl reagents on lipid flip-flop as determined by the reduction method, ROS membranes were treated with NEM, a reagent with the same

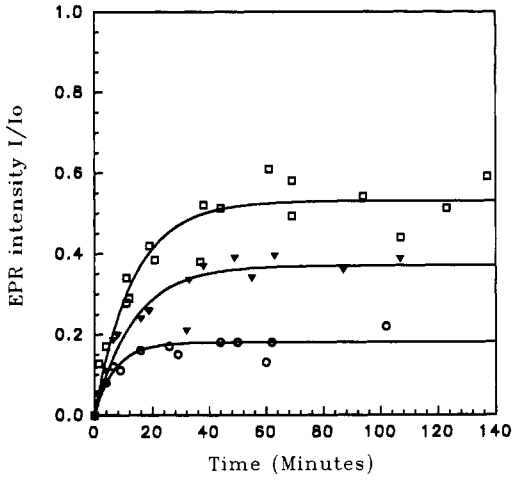


FIGURE 6: Flip-flop rate and transmembrane distribution of PCSL (□), PESL (▼), and PSSL (○) in native ROS membranes measured by the ascorbate reduction method. Spin-labeled lipids were first incorporated into the outer monolayer and incubated in 100 mM HEPES, pH 7.4, at 39 °C. Aliquots of samples were taken out at indicated times and rapidly cooled to 1 °C. The fraction of internalized spin-labeled lipid was analyzed as described in Figure 5. For each class of lipids, three independent experiments on different membrane preparations are shown. The solid lines are obtained by fitting the data to eq 5.

Table I: Equilibrium Populations and Half-Times for Flip-Flop of Spin-Labeled Lipids at 39 °C in Disc Membranes As Determined by the Ascorbate Reduction Method

lipid	percentage in inner monolayer	$\tau_{1/2}$ (min)
PCSL	53 ± 6	9.1 ± 0.9
PESL	37 ± 4	9.2 ± 1.2
PSSL	18 ± 2	5.8 ± 0.6

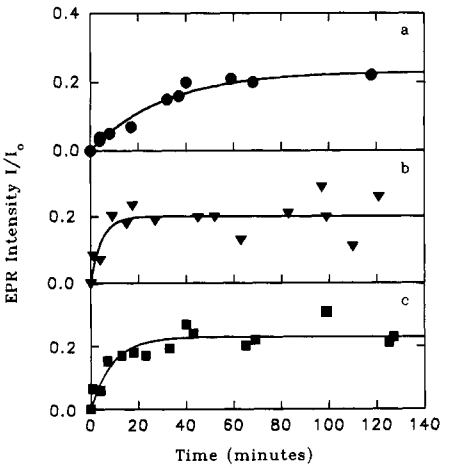


FIGURE 7: Flip-flop of (a) PCSL, (b) PESL, and (c) PSSL in NEM-treated disc membranes. The experimental conditions were the same as in Figure 6, except that the disc membranes were premodified with NEM by incubating the membranes with 2 mM NEM (greater than 20 times the rhodopsin concentration) at 21 °C pH 7.4, for 3 h.

reactive functionality as MSL. As shown in Figure 7a, the effect of prior treatment with NEM is to significantly slow down the transmembrane migration process of PSSL. Fitting of the data in Figure 7a to eq 5 shows that 23% ± 2% of PSSL residues in the inner monolayer at equilibrium, and $k_i + k_o = 0.028 \pm 0.004$, corresponding to a half-time for the flip-flop of 26 ± 4 min. Thus the transmembrane migration rate and equilibrium distribution of PSSL as determined by the ELDOR and reduction methods are the same within experimental error when membranes used in the reduction methods

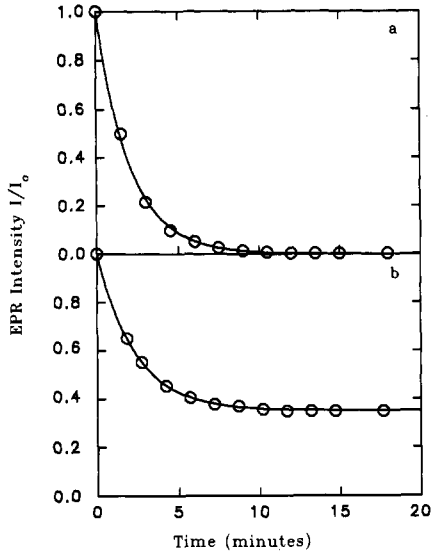


FIGURE 8: Reduction of PCSL in egg PC vesicles by 50 mM ascorbate. The vesicles were made by extrusion and were approximately 500 Å average diameter. (a) PCSL was added at a molar concentration of 1% of the total lipid, incubated at 39 °C for 2 h, cooled to 1 °C, and reduced with ascorbate as described in Figure 5. The points represent the EPR signal amplitude of the spin-labeled lipid as a function of time during the reduction. The solid line was obtained by fitting the data to a pseudo-first-order reduction (half-life 1.3 min). (b) PCSL (2% of the total lipid) was premixed with egg PC and the mixture extruded to form vesicles as described in (a). The vesicles were cooled to 1 °C and reduced with ascorbate as in Figure 5. The solid line was obtained by fitting the data to a pseudo-first-order reduction of 64% of the spin (half-life 1.3 min) with the remainder being nonreducible.

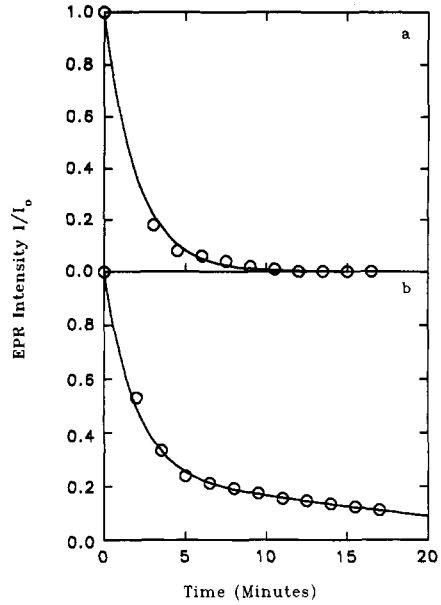


FIGURE 9: Reduction of PCSL in reconstituted membranes with 100:1 molar ratio of egg PC to rhodopsin. The vesicles have an average diameter of about 350 Å. (a) PCSL was added to the outer monolayer of reconstituted vesicles and incubated at 39 °C for 2 h. The vesicles were cooled to 1 °C and reduced with ascorbate according to the legend of Figure 5, and the EPR signal amplitude was followed as a function of time. The solid line was obtained by fitting the data to a single pseudo-first-order reduction, which gives a rate constant of 10 M^{-1} s^{-1}. (b) PCSL (2% of the total lipid) was premixed with egg PC and rhodopsin and the membranes were reconstituted. The vesicles were cooled to 1 °C and reduced with ascorbate as in Figure 5, and the EPR signal amplitude was followed as a function of time. The solid line was obtained as described in the Appendix.

are pretreated with maleimide. Apparently, MSL in the ELDOR experiment acts in a manner analogous to NEM.

As shown in Figure 7b,c, pretreatment with NEM also has a dramatic effect on the flip-flop of PCSL and PESL. A rapid component of flip-flop is observed that leads to approximately 23% and 20% of PCSL and PESL protected, respectively. One interpretation of this data is that NEM treatment modifies the membrane structure to produce an altered transmembrane equilibrium distribution of the labeled lipids. However, if PCSL is allowed to first reach the equilibrium state given in Table I and subsequently treated with NEM, the equilibrium state is unchanged.

In light of the data in Figure 7b,c, it is clear why the ELDOR approach revealed no time dependence to the concentration of PESL and PCSL in the outer monolayer; due to experimental constraints, the first data point in the ELDOR experiment is obtained at 10 min. As can be seen in Figure 7, the flip-flop process is essentially complete for PESL and PCSL at this time.

Transmembrane Migration and Equilibrium Asymmetry in Phospholipid Vesicles and Rhodopsin Reconstituted Membranes. The rapid flip-flop and the sensitivity to NEM and MSL suggest a protein-mediated process. If so, rhodopsin itself may be involved either in a transport role or simply by increasing the passive diffusion rate. To investigate this possibility, the flip-flop and asymmetry were investigated by the reduction method in reconstituted membranes containing highly purified rhodopsin as the only known protein. As a control, pure lipid vesicles were also investigated and will be discussed first.

Figure 8a shows the ascorbate reduction at 1 °C of PCSL that was previously incubated with extruded egg PC vesicles at 39 °C for 2 h. The label is completely reduced in a process accurately described by a homogeneous pseudo-first-order reaction (solid line). This could result either from the absence of flip-flop to the inner monolayer or from a vesicle population extremely leaky to ascorbate. To distinguish these possibilities, another experiment was carried out in which the PCSL and freeze–thawed with the egg PC suspension prior to vesicle formation so that the label was in both monolayers. Reduction with ascorbate at 1 °C now reveals a stable protected population corresponding to PCSL in the inner monolayer (Figure 8b). Thus, the vesicles are not leaky to ascorbate at 1 °C, and together these results demonstrate that PCSL does not flip-flop appreciably at 39 °C in pure lipid vesicles over the course of 2 h. The fraction of PCSL in the inner monolayer at equilibrium is about 36%, compared to 39% expected theoretically for 500-Å diameter vesicles.

Figure 9 shows the corresponding experiments for vesicles containing purified rhodopsin at the same density as in the native membranes (100:1 lipid/rhodopsin mole ratio). These vesicles contain egg PC as the sole lipid and have an average diameter of about 350 Å. Figure 9a shows the ascorbate reduction at 1 °C of PCSL that was previously added to the external monolayer of reconstituted vesicles and incubated at 39 °C for 2 h. As for the pure lipid vesicles, the entire complement of label is reduced in a single-exponential process (solid curve, Figure 9a). Again, this could be due either to the absence of flip-flop of the PCSL or to vesicles very leaky to ascorbate. If PCSL is added to the lipid in detergent prior to reconstitution, the label resides in both the inner and outer monolayer. As with the pure lipid system, ascorbate reduction in these vesicles has two distinct phases, corresponding to the label in the outer and inner monolayers (Figure 9b). This latter result demonstrates that the rhodopsin-containing vesicles have a distinguishable inner and outer monolayer but that ascorbate leakage is significantly higher than for the pure lipid vesicles. However, the ascorbate leakage is still sufficiently slow to conclude that the rapid reduction in Figure 9a is due not to ascorbate permeation but to the absence of

flip-flop of PCSL. Thus the lipid migrates much more slowly in rhodopsin reconstituted vesicles than in the disc membrane. Over a period of 2 h, there is no detectable flip-flop of PCSL in the reconstituted vesicles, whereas the equivalent process in the disc membrane has a half-life of about 26 min. This indicates that rhodopsin alone is not capable of catalyzing such a rapid flip-flop in these vesicles.

From a comparison of Figures 8 and 9, it is apparent that the presence of rhodopsin induces a significant ascorbate permeability in the vesicles, even at 1 °C. As a result, the nitroxide in the inner monolayer is also reduced significantly over the time course of the experiment, and it is not possible to simply extrapolate the slow process to time zero to determine the equilibrium fraction of spin in the inner monolayer. However, the data can be analyzed by integration of the appropriate differential rate laws as described in the Appendix. Using the rate constant for the pseudo-first-order reduction at the outer monolayer determined from Figure 9a, the theoretical fit to a two-reduction process is shown by the solid line in Figure 9b. The only adjustable parameters were the rate constant for ascorbate leakage and the fraction of label on the outside surface at time zero. The fit is excellent and indicates that approximately 20% of the PCSL resides on the inner surface of the vesicle at equilibrium. This is considerably less than the 34% expected for an unbiased population in these 350-Å vesicles. However, it is known that rhodopsin is also not symmetrically disposed in vesicles of this size (Fung & Hubbell, 1978b), and a surface potential asymmetry exists (Tsui et al., 1990). Thus in general, an unbiased distribution is not expected.

DISCUSSION

The spin-labeled lipids PCSL, PESL, and PSSL accurately reflect the flip-flop and equilibrium distribution of the corresponding native lipids in other systems (Tilley et al., 1986; Seigneret & Devaux, 1984; Daleke & Huestis, 1985), and we assume that to be the case for the disc membrane. On the other hand, Middelkoop et al. (1986) have reported a dependence of the flip-flop rate on chain composition, and it is possible that the kinetics of spin-label transport may differ quantitatively from other molecular species of the same headgroup class. The BSA extraction procedure to determine the asymmetry of the labeled lipid is considerably more convenient than ascorbate reduction but did not lead to quantitative extraction of the labels from the disc membranes. The partition coefficient of the spin labels between BSA and the disc membrane was too much in favor of the highly fluid lipid environment of this membrane. Thus ascorbate reduction was used in all experiments.

An important result of this work is that all three species of lipids flip-flop rapidly at physiological temperature, and the process is not ATP-driven. The mechanism could be simple or facilitated diffusion, but the NEM sensitivity suggests the latter. A similar non-ATP-driven rapid flip-flop has been found in ER membranes (Bishop & Bell, 1985; Herrmann et al., 1990). The existence of a non-energy-requiring flip-flop mechanism implies that the distribution of lipids is determined by thermodynamic equilibrium considerations, and any asymmetry must arise from other stable asymmetries inherent to the system. In the disc membrane, rhodopsin is asymmetrically oriented and present in large amounts. On the basis of this stable asymmetry, Hubbell (1990) proposed a transbilayer coupling model that predicted a significant asymmetry in PS with approximately 80% of that lipid on the outer, cytoplasmic surface. The results presented here place 82% of the PS on the outer surface, in excellent agreement with the model. The transbilayer coupling model further predicts an equilibrium asymmetry of PE and PC stabilized by the asymmetry in PS through nonideal solution interactions. Although the quantitative predictions of the theory are less accurate for these lipids due to lack of sufficient information on the magnitude of lipid–lipid interactions and composition, it does predict an asymmetry of PE and PC in qualitative agreement with the measurements presented here. For example, 63% of the PE and 47% of the PC are found in the outer monolayer compared to 70% and 30% predicted, respectively. These values are quite close considering that the predicted values for PE and PC were computed by ignoring all other lipid species of the disc membrane.

The rapid flip-flop found for the lipids in the disc membrane at 39 °C precludes the use of chemical modification methods for determination of lipid asymmetry, at least at that temperature. The activation energy measured for phospholipid transmembrane diffusion in pure lipid vesicles is about 20 kcal/mol (Kornberg & McConnell, 1971). With this activation energy, an upper limit to the half-time for flip-flop at 25 °C would be about 50 min. Facilitated diffusion should have a lower activation energy. Thus chemical modification reactions to determine lipid asymmetry that last longer than about 10 min will likely yield erroneous results. Most studies of lipid asymmetry in the disc membrane are based on chemical modification and/or lipid hydrolysis. In view of the present results, it is perhaps not surprising that the literature contains a wide variation in the reported values of lipid asymmetry in this membrane. The most recent study of this type in the disc membrane was that by Miljanich et al. (1981) using imidoesters as reactive probes. These authors reported that a minimum of 73–87% of the PE and 77–88% of the PS was in the disc outer monolayer. The modification reactions lasted 4–5 h. Note that the effect of flip-flop is to overestimate the amount of lipid in the external monolayer, consistent with the higher values found in that study compared with the results presented here. Smith et al. (1977) reported that 100% of the aminophospholipids were exposed on the outer membrane surface, a result almost certainly affected by lipid flip-flop.

The effect of NEM on the flip-flop reaction is dramatic (Figure 7) and distinguishes two classes of behavior, one for PS and the other for PC and PE. In the case of PS, the rate of flip-flop is slowed by a factor of about 2. In addition, there appears to be some effect on the equilibrium asymmetry, but the difference is not much outside the experimental errors. The PS flip-flop kinetics can be accurately fit by a simple homogeneous first-order process. In the case of PC and PE, NEM treatment apparently permits the rapid flip-flop of only about 20% and 23% of the spin-labeled PC and PE, respectively, with the remaining external population being stable for at least 2 h. These results suggest a possible heterogeneity in the vesicle population. For example, a major vesicle population with a transport system for PE and PC blocked by NEM and a subpopulation with an NEM-insensitive system could account for the data. A simple calculation indicates that about 20% of the vesicles would have to contain an NEM-insensitive system for PE and PC. We have no information regarding the possible identity or origin of a minor vesicle population. However, the high purity of the preparation makes it unlikely that it is due to a contamination from a foreign membrane.

Another explanation to be considered for the data in Figure 7 is that all of a putative transporter population is blocked by NEM, but some fraction of the externally located spin label is physically protected from reduction, say by being trapped in gel-phase lipid at the low temperature of the ascorbate

reduction. Indeed, this is believed to occur at 4 °C in platelet membranes (Sune et al., 1987), and about 20% of the external spin label cannot be reduced. It has been reported that a gel phase is present in the inner monolayer of disc membranes (Sklar & Dratz, 1980) below about 30 °C. However, this is in the inner monolayer, and could not trap and protect spin label in the outer monolayer. Furthermore, the spin labels are completely and rapidly reduced by ascorbate immediately after their addition to disc membranes (see Figure 7, $t = 0$). Finally, the EPR line shape of the spin-labeled lipids in disc membranes at 1 °C reflects a homogeneous motional population, and this remains constant during the reduction. This is inconsistent with the notion of some 20% being trapped in a gel phase and the other 80% being accessible.

The disc vesicles that were the object of the present and other studies of lipid asymmetry are in a physical state quite different than that in the native rod outer segment. Specifically, the discs in the photoreceptor cell are flattened, with the intradiscal surfaces on the order of 25 Å apart. Such close apposition implies a strong interaction between surfaces that could lead to significant effects on lipid asymmetry. In contrast, the isolated disc membranes prepared from osmotically lysed ROS are roughly spherical vesicles. The native disc also has a steep concentration gradient of Ca^{2+} across the membrane, high in the intradiscal space. This situation would be expected to alter surface potential asymmetry with a concomitant rearrangement of charged lipids (Hubbell, 1990). In fact, Schnetkamp (1985) has provided indirect evidence that PS may be localized on the inner monolayer of intact disc membranes. Thus, the lipid asymmetry in the native stacked disc arrangement may be very different than in the isolated disc vesicles. Further studies of the intact membranes are certainly warranted.

ELDOR spectroscopy provides an elegant and sensitive means of directly monitoring the collision rate of two spin-label populations. Since the collision rate of one spin with another is proportional to the local concentration, ELDOR provides a technique for directly monitoring local concentration, whereas conventional spectroscopy simply monitors total numbers of spins. Thus it is ideally suited to determination of the distribution of spins within a system. That is the use to which it is applied here, to determine the transmembrane distribution of a spin-labeled lipid. This is a general approach that could be used to monitor the distributions of a charged spin label for the determination of transmembrane potential or asymmetry in surface potentials, as examples. In principle, EPR line broadening or fluorescence quenching could be used to accomplish the same end, but as previously discussed the ELDOR method is sensitive to much lower collision frequencies (Hyde & Feix, 1989; Shin & Hubbell, 1992).

Application of the ELDOR approach as described requires that a stable reference nitroxide be provided on one membrane surface. In the present studies, that reference was provided by attaching a nitroxide to a rhodopsin sulfhydryl group located near the membrane solution interface. This offers a convenient and highly stable reference. However, the sensitivity of the transport process to maleimide sulfhydryl reagents greatly limits this implementation of the ELDOR strategy. Nevertheless, the data presented clearly establish the principle of the experiment and provide an independent determination of PS asymmetry and flip-flop kinetics. The agreement between the ELDOR and ascorbate reduction data for the NEM-modified membranes is particularly gratifying since the ELDOR experiment monitors disappearance of spin from the outer monolayer, while the reduction monitors appearance in the inner monolayer.

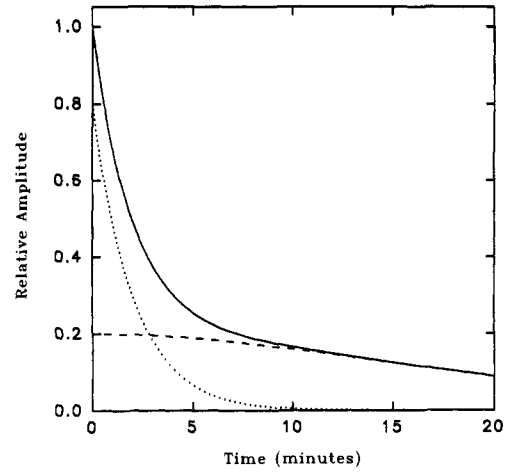


FIGURE 10: Time courses of the external nitroxide concentration (···), the internal nitroxide concentration (- - -), and the total nitroxide concentration (—) during ascorbate reduction obtained from a numerical solution to the rate laws describing the reduction/diffusion process. The particular situation shown here corresponds to that of Figure 9b. The rate constant for the nitroxide reduction at the inner or outer surface under the conditions of Figure 9 was determined to be $k_r = 10\ M^{-1}\ s^{-1}$ from fitting the data in Figure 9a, and the rate constant of nitroxide permeation was obtained as $k_1 = 0.01\ s^{-1}$ for the best visual fit.

In future implementations of the ELDOR experiment, an amphiphilic nitroxide with a low flip-flop rate will be utilized as an outer monolayer reference, thus avoiding sulfhydryl modification reactions. The instrumentation used in these experiments imposed a relatively low time resolution; it presently takes on the order of 1–2 min to obtain the collision rate at each time point. However, relatively simple instrumental improvements can shorten these times to the subsecond domain. The potential advantages of the ELDOR method over reduction or extraction schemes for investigating transmembrane distributions of lipids or other species warrants its further development.

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APPENDIX

Consider membrane vesicles with nitroxides residing in both the inner and outer monolayers and with no flip-flop. Addition of ascorbate at time zero in the external solution leads to reduction of both populations if ascorbate has a finite membrane permeability. The reduction is first order in both nitroxide and ascorbate (data not shown); the relevant differential rate laws are

$$\frac{-d[N_o]}{dt} = k_r[asc_o][N_o]$$

$$\frac{-d[N_i]}{dt} = k_r[asc_i][N_i]$$

$$\frac{d[asc_i]}{dt} = k_1([asc_o] - [asc_i]) - k_r[asc_i][N_i]$$

where $[N_i]$ and $[N_o]$ are the effective concentrations of nitroxide on the interior and exterior of the vesicle, respectively, $[asc_i]$ and $[asc_o]$ are the corresponding concentrations of

ascorbate, and k_r and k_l are the rate constants for reduction and ascorbate diffusion across the membrane, respectively. The external concentration of ascorbate is in great excess over the nitroxide and may be taken to be constant. Thus the reduction of the N_o is pseudo-first-order, as mentioned above. The fit of the data in Figure 9a to a single exponential gives a rate constant of 10 M^{-1} s^{-1}. Using this value and selected initial values of $[N_i]$ and k_l, the differential rate laws can be numerically integrated to determine the time dependence of $[N_i]$. Numerical integration was carried out using the fourth-order Runge–Kutta method (Press et al., 1986). An excellent fit of the data to this model is obtained with an initial value of $[N_i]$ corresponding to 20% of the total spin and a value of k_l = 0.01 s^{-1} (Figure 9b).

Figure 10 shows an analysis of the reduction of nitroxide for the experiment of Figure 9b. Note that the internal nitroxide does not decay according to an exponential process but shows a lag phase during initial ascorbate entry. Thus, analysis of such data according to pseudo-first-order processes leads to significant errors in the estimation of internal populations. Simulations show that, for reductions of internal nitroxide as slow as that shown in Figure 5, a linear extrapolation to zero leads to very little error in the equilibrium population.

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