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# A New Electron Spin Resonance Assay for Membrane Asymmetry and Entrapped Volume of Unilamellar Lipid Vesicles Based on Photoreduced Flavin Adenine Dinucleotide†

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ABSTRACT: A new ESR assay has been developed for the characterization of unilamellar lipid vesicles. It is based on the reduction by photogenerated $FADH_2$ of amphiphilic spin-labels having the spin in the polar group. $FADH_2$ is generated in situ under anaerobic conditions from its oxidized form (FAD) by photoreduction in the presence of excess EDTA as the reducing agent. Photoreduction is induced by exposing the FAD/EDTA mixture to white light of a commercial slide projector. $FADH_2$ as an impermeable agent reduces spin-label molecules located on the outer layer of the bilayer that are readily accessible in a first fast reaction; spin-label located on the inner layer of the bilayer is reduced in a second slow reaction. The ESR assay is suitable for the routine characterization of unilamellar membrane vesicles: it allows the determination of the vesicle size, the entrapped volume, the bilayer asymmetry, the bilayer integrity, and the vesicle stability. The ESR assay developed is of general applicability: it can be used with charged and uncharged bilayers which may be labeled with either neutral or charged spin-labels. An assessment of the new ESR assay is given in comparison to the existing ascorbate method which uses sodium ascorbate as the reducing agent. Various other potential reducing agents for spin-labels have been tested and found unsuitable for the ESR assays discussed here.

A number of physicochemical methods have been employed and also new ones have been developed in the past decades that are suitable for the characterization of lipid bilayers and membrane vesicles. Among these methods ESR spin-labeling has been used succesfully to determine the fraction of spin-labeled lipid molecules located on the external surface of lipid bilayers and, related to it, the asymmetry in the transverse lipid distribution. Other applications of ESR techniques comprise the determination of the transverse bilayer motion or "flip-flop" of spin-labeled molecules (Kornberg & McConnell, 1971), the thermodynamic stability and integrity of bilayers of multilamellar and unilamellar vesicles (Strauss & Hauser, 1986), and the volume fraction entrapped in lipid vesicles (Marsh et al., 1976; Marsh & Watts, 1981). This entrapped volume is directly related to the encapsulation properties of lipid and membrane vesicles. Its routine determination is important when vesicles are used as carrier systems, e.g., in drug delivery.

All the spin-labeling methods mentioned above have in common that the spin-label or at least part of it is reduced to the corresponding hydroxylamine or other nonparamagnetic compounds under the action of the reducing agent sodium ascorbate (Smith et al., 1976; Marsh & Watts, 1981):

$$2{>}N\text{—}\dot{O} + Na^+ \text{ ascorbate} + H^+ \rightarrow 2{>}N\text{—}OH + Na^+ + \text{dehydroascorbate} \quad (1)$$

The use of sodium ascorbate as the reducing agent in ESR

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assays is based on the observation that egg phosphatidylcholine bilayers are practically impermeable to sodium ascorbate at 0 °C (Kornberg & McConnell, 1971). The spin-labeled lipids used in this kind of ESR assays have the spin group in the polar group or in the hydrophobic region close to the polar group so that it is readily accessible to the sodium ascorbate. The determination of the internal volume, frequently referred to as the "protective volume" assay, involves the reduction at 0 °C of a water-soluble spin-label (e.g., Tempocholine)[1] present in the external medium with excess ascorbate. The ESR spectrum recorded under these conditions then arises only from spin-label entrapped within the liposomes and not accessible to the added ascorbate. By a slight modification of the experimental protocol, the permeability of the lipid bilayer to water-soluble spin-labels can also be measured (Marsh & Watts, 1981).

The ESR assays based on the use of sodium ascorbate as the reducing agent have serious shortcomings, and their application is therefore limited. The method was originally developed for neutral (isoelectric) phosphatidylcholine bilayers doped with spin-labeled phosphatidylcholine. Under these conditions satisfactory results are obtained. In contrast, the kinetics of the reduction by ascorbate of negatively charged spin probes incorporated in either phosphatidylcholine bilayers or negatively charged bilayers are too slow for all practical purposes. Furthermore, at temperatures above 0 °C lipid bilayers and membranes become permeable to sodium ascorbate, and the results have to be interpreted with great care.

Here we introduce a new ESR assay that is more versatile and therefore of wider applicability than the ascorbate method. It is based on the use of reduced flavin adenine dinucleotide ($FADH_2$) as the reducing agent. Under aerobic conditions the flavin is present in its oxidized form as evident by the deep yellow color of its solution. Under anaerobic conditions, over a wide pH range, photoreduction of flavins by excess EDTA can be induced by exposing a solution of EDTA and FAD to white light [Hemmerich, 1976; Massey et al., 1978; reviewed by Müller et al. (1988)]:

$$\text{FAD} + \text{EDTA} \xrightarrow{+H_2O} \text{FADH}_2 + \text{ethylenediaminetriacetate} + CH_2O + CO_2 \quad (2)$$

The formation of the reduced form, $FADH_2$, is indicated by fading of the yellow color of FAD. The concentrations of FAD and $FADH_2$ can be determined by measuring optical densities at 449 nm. Gassing this pale yellow solution of $FADH_2$ with oxygen reproduces the deep yellow color of FAD. FAD is a highly polar molecule. According to its redox potential, it should be an efficient reducing agent for spin-labels and hence a good replacement of ascorbate in the ESR assays discussed above. Here we discuss its advantages and disadvantages with respect to the sodium ascorbate method.

## Materials and Methods

Egg phosphatidylcholine (EPC) and ox brain phosphatidylserine (PS) were purchased from Lipid Products (South Nutfield, U.K.), and 1,2-dipalmitoyl-*rac*-phosphatidylethanolamine (DPPE) was from Fluka (Buchs, Switzerland). 4-(*N*,*N*-Dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT 16), succinimidyl 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylate, and Tempocholine were obtained from Molecular Probes (Eugene, OR). 4′,4′-Dimethylspiro[5α-cholestane-3,2′-oxazolidine]-*N*-oxyl (=3-doxyl-5α-cholestane) was from Aldrich (Steinheim, FRG). Ascorbic acid, L-ascorbic acid 6-palmitate, and the disodium salt of flavin adenine dinucleotide (FAD) were purchased from Fluka; the disodium salt of ethylenediaminetetraacetic acid (EDTA) was from Siegfried (Zofingen, Switzerland). Hydrazine sulfate, phenylhydrazine, sodium thiosulfate, and sodium sulfite were obtained from Fluka, sodium dithionite was from Merck (Darmstadt, FRG), glutathion and the reduced form of nicotinamide adenine dinucleotide (NADH) were from Sigma (St. Louis, MO), and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was from Boehringer (Mannheim, FRG) and Fluka. The sodium salt of ascorbic acid was made by adding stoichiometric quantities of NaOH to ascorbic acid. The phospholipids and CAT 16 were used without further purification; they were found to be pure by TLC standard. All other chemicals were of analytical grade. Solutions and lipid dispersions were made with glass-distilled water. Unless stated otherwise, the buffer used throughout this work was 2.5 mM sodium phosphate, pH 7.1, containing 0.14 M NaCl and 0.02% $NaN_3$.

*Synthesis of 1,2-Dipalmitoyl-N-(2,2,5,5-tetramethyl-1-oxy-3-pyrroline-4-carbonyl)-rac-phosphatidylethanolamine (PESL).* The synthesis of PESL was carried out according to a modified procedure described by Martin et al. (1981). DPPE (38 mg) was dissolved in $CHCl_3$ and mixed with 19.6 mg of succinimidyl 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylate dissolved in $CHCl_3$ and 9.7 μL of triethylamine freshly distilled over ninhydrine. The final concentrations in $CHCl_3$ of DPPE, spin-label, and triethylamine were 29 mM, 37 mM, and 37 mM, respectively. The reaction mixture was stirred at 45 °C under an argon atmosphere for 5 h. TLC analysis was used to monitor the progress of the reaction which had gone to completion after about 3–4 h. To the $CHCl_3$ solution (1.9 mL) an equal volume of $CH_3OH$ was added, and the organic phase was washed two times with 10 mL of 1% NaCl solution. The resulting organic phase was taken to dryness on the rotary evaporator, the dry residue was dissolved in 0.5 mL of $CHCl_3$ and applied to a silica gel column (10 × 2.5cm). The silica gel 60 (70–230-mesh ASTM, Merck) was activated before use at 160 °C overnight. The column was eluted with $CHCl_3$ containing increasing quantities of $CH_3OH$ starting with a volume ratio of 40:1. The spin-labeled DPPE (PESL, Figure 1) was eluted with $CHCl_3/CH_3OH$ mixtures ranging between 15:1 and 8:1 (by volume). It was pure by TLC standards (silica gel 60 $F_{254}$, Merck: $CHCl_3/CH_3OH/H_2O/CH_3COOH$, 65:50:4:1 by volume; $R_f = 0.6$). Spectral data: fast atom bombardment mass spectroscopy (negative ion, matrix: *o*-nitrobenzyl alcohol), mass over charge ($m/z$) = 858 (41%), 857 (100%, molecular ion), 856 (65%), 855 (16%), 841 (8%), 825 (14%), and several peaks at <500 from the matrix; UV/vis spectrum of PESL (recorded in $CH_3CH_2OH$ at 0.5 mM), one peak at $\lambda_{max}$ ($\epsilon$) = 277 nm (~520), tailing up to 400 nm.

*Preparation of Spin-Labeled Small Unilamellar Phospholipid Vesicles.* Appropriate amounts of phospholipid (either EPC or PS) and spin-label (either CAT 16 or PESL) dissolved in 2:1 $CHCl_3/CH_3OH$ (by volume) were mixed in a 25-mL round-bottom flask. The molar ratio of phospholipid/spin-label was 100:1 unless otherwise stated. The organic

[1] Abbreviations: EPC, egg phosphatidylcholine; PS, ox brain phosphatidylserine; DPPE, 1,2-dipalmitoyl-*rac*-phosphatidylethanolamine; CAT 16, 4-(*N*,*N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; Tempocholine, 4-[*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonio]-2,2,6,6-tetramethylpiperidine-1-oxyl; PESL, 1,2-dipalmitoyl-*N*-(2,2,5,5-tetramethyl-1-oxy-3-pyrroline-4-carbonyl)-*rac*-phosphatidylethanolamine; FAD, flavin adenine dinucleotide disodium salt; EDTA, disodium salt of ethylenediaminetetraacetic acid.

solvent was evaporated in a Büchi rotary evaporator, and the lipid film thus deposited on the glass wall of the round-bottom flask was dried in vacuo. The dry lipid film was dispersed in buffer by hand-shaking with a few glass beads to yield typically 2% lipid dispersions (0.027 M phospholipid); 3 mL of the resulting phospholipid dispersion was subjected to ultrasonication with a Branson B-30 sonicator with a microtip under standard conditions as described before (Hauser, 1971). The sonicated dispersion was centrifuged at 12000*g* for 10 min to remove titanium particles and possibly large multilamellar phospholipid structures (not more than 2% of the total lipid dispersed).

*ESR Assays.* (*A*) *Reduction with Ascorbate.* Both the sonicated, spin-labeled phospholipid dispersion and the sodium ascorbate stock solution were adjusted to the desired temperature and then mixed. Unless otherwise stated, the final concentration of sodium ascorbate was 25 mM. The mixture was filled into glass capillary tubes of 1-mm internal diameter, and ESR spectra were recorded as a function of time. That the sodium ascorbate concentration was sufficient was shown by solubilizing EPC vesicles (labeled with CAT 16) with sodium cholate (23 mM). In the resulting mixed micelles 25 mM ascorbate reduced all spin-label instantaneously.

(*B*) *Reduction with Photogenerated $FADH_2$.* Appropriate volumes of the sonicated, spin-labeled phospholipid dispersion were mixed with appropriate volumes of EDTA and FAD stock solutions in the same buffer to yield the desired final concentrations. The mixture was cooled to 0 °C, flushed with $N_2$ for about 3 min, and filled into the ESR capillary. The photoinduced reduction (Massey et al., 1978) was achieved by exposing the capillary, thermostated at the desired temperature, to white light of a slide projector (Pradovit Color 250 with a 24V/250W bulb from Leitz, Wetzlar, FRG). The lamp was not focused; instead, the total dispersion in the capillary tube was illuminated. The ESR capillary was inserted immediately into the thermostated ESR cavity, and the ESR spectrum was recorded.

(*C*) *Determination of the Internal Volume of Small Unilamellar Vesicles* (*Protective Volume Assay*). Sonicated EPC dispersions in buffer containing 1.0–1.3 mM Tempocholine were prepared as described above. The addition of Tempocholine to the dispersion medium ensured that the spin-label concentration was equilibrated between the external medium and the internal cavity of the unilamellar EPC vesicles. To the ready-made sonicated EPC vesicle dispersion excess sodium ascorbate (25 mM) was added, or alternatively a mixture of EDTA (12.5 mM) and FAD (0.016–0.031 mM), and photoreduction was induced as described above. The assay was carried out at 21 °C.

(*D*) *ESR Measurements.* ESR spectra were run at 9.2 GHz with a Varian X-band spectrometer (Model E-104A) fitted with a variable-temperature control. Temperatures were monitored with a thermocouple before and after each spectrum was recorded and were accurate to ±0.5 °C. Since line-height measurements were in good agreement with intensity values determined by double integration, line heights of the low-field and center line of the ESR spectra were measured routinely and used as a first approximation of signal intensities. Double integration of the ESR spectra was carried out on a Bruker BNC-12 computer interfaced with the Varian spectrometer.

## Results

The chemical structure of the spin-labels used in this work is given in Figure 1. The ESR spectra of sonicated EPC dispersions labeled with PESL and CAT 16 are shown in spectra A and B of Figure 2, respectively. The line shape of

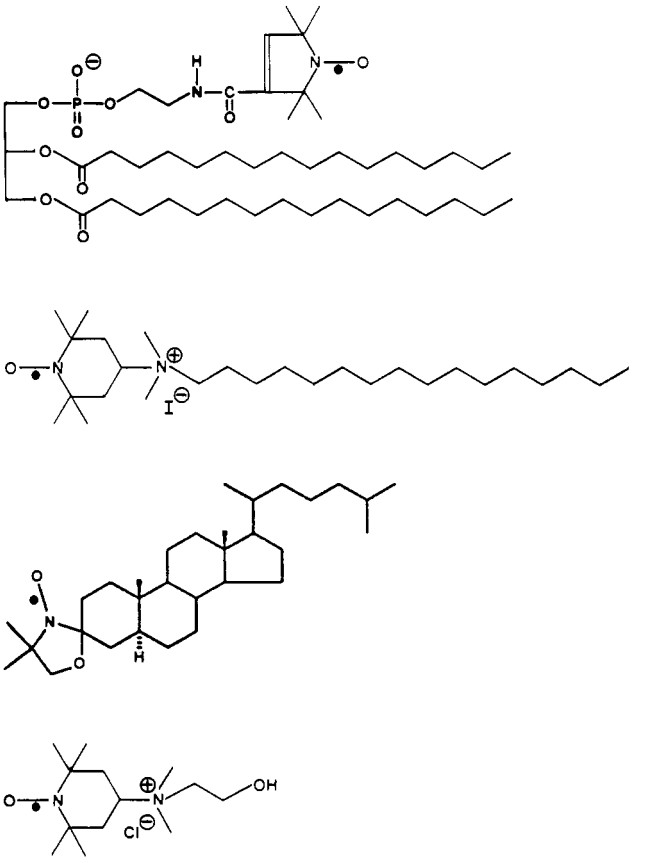

FIGURE 1: Chemical structure of spin-labels used in this work: PESL, CAT 16, 3-doxyl-5$\alpha$-cholestane, and Tempocholine (top to bottom).

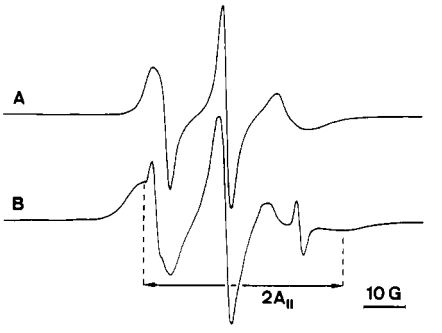


FIGURE 2: ESR spectra of sonicated EPC dispersions labeled with PESL (A) and CAT 16 (B). Sonicated, spin-labeled EPC dispersions [33 mM for (A); 26 mM for (B); [EPC]/[spin-label] = 100 for both dispersions] in buffer were made as described under Materials and Methods. ESR spectra were recorded at 21 °C.

both ESR spectra (Figure 2) is indicative of rapid but anisotropic motion of the spin group. The anisotropy of motion appears to be greater for CAT 16 (Figure 2B) as indicated by the hyperfine splitting $2A_{\parallel}$. The narrow three-line spectrum that is superimposed on the anisotropic component of the CAT 16 spectrum probably arises from free spin-label present in the aqueous solvent. Similar ESR spectra to those shown in Figure 2 were obtained from sonicated PS vesicles labeled with PESL or CAT 16 (data not shown). The effect of two reducing systems, sodium ascorbate or L-asorbic acid 6-palmitate and EDTA/FAD/visible light on different spin-labeled phospholipid dispersions is shown in Figures 3–7.

*Reduction of Spin-Labels by Sodium Ascorbate.* Four different sonicated, spin-labeled phospholipid dispersions were treated with sodium ascorbate: EPC labeled with PESL or CAT 16 and PS labeled with PESL or CAT 16. The sonicated, spin-labeled dispersions of both EPC and PS were shown

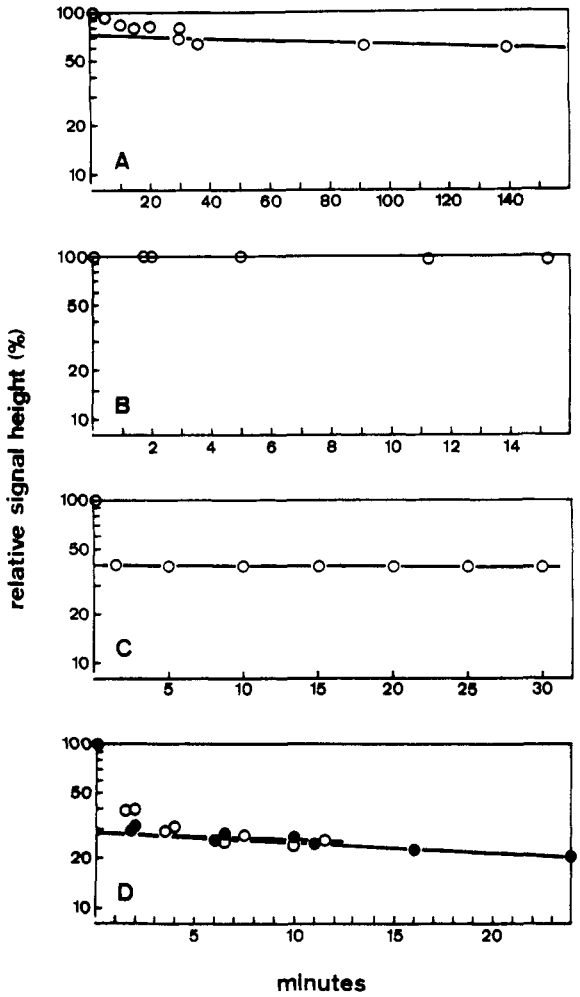


FIGURE 3: Kinetics of the reduction by sodium ascorbate of four sonicated, spin-labeled phospholipid dispersions: (A) EPC (26 mM) labeled with PESL; (B) PS (30 mM) labeled with PESL; (C) EPC (26 mM) labeled with CAT 16; (D) PS (24 mM) labeled with CAT 16. The phospholipid/spin-label molar ratio was 100. All experiments were carried out at 0 °C (○). The reduction of CAT 16 incorporated in PS bilayers (10 mM) was also carried out at 22 °C (●) (D). Sonicated, spin-labeled phospholipid dispersions were made as described under Materials and Methods. The sodium ascorbate concentration was 25 mM in all experiments. Normalized signal heights of the low-field or center line of the ESR spectrum remaining after exposure of the phospholipid dispersion to ascorbate were plotted as a function of the reaction time, i.e., the time the phospholipid dispersion was exposed to the reducing agent sodium ascorbate.

by gel filtration on calibrated Sepharose 4B columns to consist mainly of small unilamellar vesicles of diameter of 20–25 nm [data not shown; cf. Huang (1969), Hauser (1971), and Hauser and Phillips (1973)]. The kinetics of the reduction by sodium ascorbate of the four spin-labeled phospholipid dispersions were compared (Figure 3). The reduction was treated as a pseudo-first-order reaction, and the data were plotted accordingly. The resulting semilogarithmic plots shown in Figure 3 are biphasic except for the reduction of PESL incorporated into PS bilayer vesicles (Figure 3B). In this experiment no reduction of the spin-label was observed at last over a period of 15 min (see Discussion). With all other samples reduction of the spin-label occurred, and the second slow phase is represented by good straight-line relations (Figure 3). Extrapolation of the straight lines of this second slow phase (Figures 3 and 4) to zero time gives an estimate of the spin-label present in the inner monolayer of the bilayer (see Discussion). Provided the spin-label distribution is random between the two monolayers of the bilayer, this estimate is

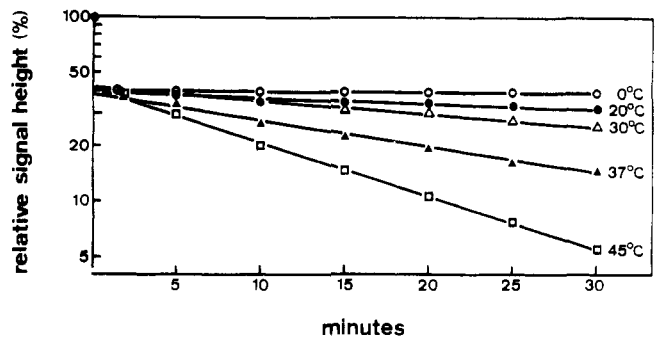


FIGURE 4: Temperature dependence of the reduction of CAT 16 incorporated into bilayers of unilamellar EPC vesicles, with sodium ascorbate. Sonicated, spin-labeled phospholipid dispersions were made as described under Materials and Methods. Signal heights of the low-field line of the ESR spectrum were normalized and plotted as a function of the reaction time, i.e., the time the phospholipid dispersion was exposed to 25 mM sodium ascorbate. Temperatures at which the reduction was carried out are given on the right of each plot.

identical with the fraction of phospholipid molecules forming the inner monolayer (=internal phospholipid). Values obtained by this extrapolation procedure are listed in Table I. The following points are obvious from an inspection of Table I and Figures 3 and 4. The ascorbate method gives satisfactory results with isoelectric EPC bilayers and probably with negatively charged PS bilayers when labeled with the positively charged spin probe CAT 16. At 0 °C the rate of reduction in the second slow phase is insignificant for EPC bilayers so that the time dependence of the remaining ESR signal intensity is negligible at least over a period of 30 min (Figures 3C and 4). As evident from these two figures, reasonably accurate values for the fraction of internal phospholipid can be obtained by measuring signal intensities 15–20 min after ascorbate addition. The fraction of internal phospholipid thus derived is about 30–40% (Table I). This value is consistent with the average diameter and the dimension of the phospholipid bilayer of small unilamellar vesicles produced by sonication. In contrast, the kinetics of the reduction by ascorbate of PESL incorporated into EPC bilayers were too slow to give meaningful results. After treatment with ascorbate for about 15 min only 15–20% of PESL were reduced (Figure 3A). The rate of reduction of PESL in the negatively charged bilayers of PS was even slower. After 15 min the reduction of the spin-label by ascorbate was barely detectable (Figure 3B). This is probably due to electrostatic repulsion between the negatively charged spin-label and the ascorbate anion. If the spin-label is located within a negatively charged phospholipid surface (i.e., in the presence of a negative surface potential $\psi_0$), the electrostatic repulsion is apparently enhanced.

The rate of reduction of spin-labels in the second, slow phase increased significantly with temperature. The temperature dependence of the reduction by ascorbate of CAT 16 incorporated into bilayers of small unilamellar EPC vesicles is shown in Figure 4. While the remaining ESR signal intensity due to internal spin-label was practically invariant at 0 °C over a period of 30 min, the reduction of internal spin-label became significant at temperatures of ≥20 °C. This may be due to diffusion of ascorbate through the EPC bilayer and/or "flip-flop" of the spin-label. At temperatures of ≥20 °C the measurement of ESR signal intensities 15–20 min after ascorbate addition would lead to erroneous results. In this case reliable values for the fraction of internal phospholipid can only be derived from a kinetic analysis and the extrapolation of the data to zero time. As shown in Figure 4, such an extrapolation procedure yields consistent values for the fraction of internal phospholipid even at 45 °C, i.e., under conditions

Table I: Fraction (%) of Spin-Label or Phospholipid Located in the Inner Monolayer of the Vesicle Bilayer, Pseudo-First-Order Rate Constants $k_1$, and Half-Lives $t_{1/2}$ Characterizing the Reduction of Spin-Labels

| phospholipid unlamellar vesicles | reducing agent[a] | temperature (°C) | fraction (%) of phospholipid forming inner monolayer[b] | first, fast phase[c] | | second, slow phase[c] | |
|---|---|---|---|---|---|---|---|
| | | | | rate constant, $k_1$ ($h^{-1}$) | half-life, $t_{1/2}$ (h) | rate constant, $k_1$ ($h^{-1}$) | half-life, $t_{1/2}$ (h) |
| EPC/PESL | ascorbate | 0 | nd[d] | | | | |
| | EDTA/FAD | 0 | 33 | 15.5 | 0.045 | 0.49 | 1.40 |
| | | 21 | 33 | 25 | 0.028 | 2.7 | 0.25 |
| PS/PESL | ascorbate | 0 | nd | 0.22 | 3.1 | | |
| | EDTA/FAD | 21 | 12 | 77 | 0.0090 | 1.22 | 0.57 |
| EPC/CAT 16 | ascorbate | 0 | 35 ± 5 ($n$ = 25) | | | 0.074 | 9.3 |
| | | 20 | 39 | | | 0.40 | 1.75 |
| | EDTA/FAD | 21 | 40 | 124 | 0.0056 | 16.0 | 0.043 |
| PS/CAT 16 | ascorbate | 0 | 28 | | | 0.34 | 2.0 |
| | | 22 | 28 | | | 0.71 | 0.97 |
| | EDTA/FAD | 21 | 37 | 74 | 0.0093 | 19.8 | 0.035 |

[a] The reduction of accessible spin-label was accomplished by sodium ascorbate or by photogenerated $FADH_2$ as described under Materials and Methods. [b] The values in this column were obtained by extrapolation to zero time of the straight-line portion of the curves in Figures 3–6. As mentioned in the text these straight-line portions were interpreted to represent the slow phase of the reduction of the spin-label located on the inner monolayer of the vesicle bilayer. [c] The first fast phase refers to the fast reduction of spin-label present in the outer layer of the bilayer, the second slow phase to that of the spin-label present in the inner layer of the bilayer. [d] nd (not determined) indicates that the proportion of the spin-label on the inner monolayer could not be determined because the kinetics of the reduction with sodium ascorbate were too slow and equilibrium was not reached in a reasonable period of time (cf. Figure 3A,B).

where the permeability of EPC bilayers to ascorbate and/or the "flip-flop" rate of the spin-label are appreciable.

A modification of the ascorbate method is the use of the surface active L-ascorbic acid 6-palmitate instead of the water-soluble sodium ascorbate. L-Ascorbic acid 6-palmitate was added to the sonicated, spin-labeled phospholipid dispersion as an aqueous dispersion or as a concentrated solution in ethanol. In its ability to reduce spin-labels, L-ascorbic acid 6-palmitate behaved like water-soluble ascorbic acid.

In the search for a replacement of ascorbate, several potential reducing agents were tested. Either the kinetics of the reduction of CAT 16 incorporated into bilayers of sonicated EPC vesicles were measured, or alternatively, the rate of reduction of Tempocholine dissolved in water or entrapped in the cavity of small unilamellar EPC was measured. All reagents mentioned below proved unsuccessful: the kinetics were either too slow [hydrazine sulfate, sodium thiosulfate ($Na_2S_2O_3$), sodium sulfite ($Na_2SO_3$), glutathione, nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH)] or too fast. In this group of reducing agents were compounds such as phenylhydrazine and sodium dithionite ($Na_2S_2O_4$), which gave fast monophasic reactions with EPC/CAT 16 sonicated vesicles and also with Tempocholine enclosed in small unilamellar EPC vesicles. The half-lives of these reactions at room temperature were on the order of minutes.

*Reduction of Spin-Labels by Photogenerated $FADH_2$.* In the following experiments $FADH_2$ is used as the reducing agent. The photogeneration of $FADH_2$ in the presence of EDTA was followed by UV/vis spectroscopy, e.g., 85% of 0.025 mM FAD in buffer were bleached within 35 s under our experimental conditions (see Materials and Methods). In the presence of a CAT 16 labeled EPC dispersion under the same conditions otherwise, the bleaching was considerably slower: only 75% of FAD were bleached within 60 s.

The kinetics of the reduction by $FADH_2$ of the same four sonicated spin-labeled phospholipid dispersions as discussed in Figure 3 are shown in Figure 5. Treating the reduction as a pseudo-first-order reaction, semilogarithmic plots were constructed. As shown in Figure 5, all four semilogarithmic plots are biphasic. The extrapolation of the second slow phase of reduction to zero time yields the proportion of molecules located on the inner monolayer of the bilayer. The results are included in Table I. There is good agreement between the reduction by ascorbate and the reduction with photogenerated $FADH_2$, where a comparison of the two methods is possible (see phospholipid vesicles labeled with CAT 16, Table I). The result obtained with PS vesicles labeled with PESL is exceptional: only 12% of PESL appeared to be located on the inner bilayer surface. This result could be due to an asymmetric distribution of PESL or alternatively to the PS bilayer becoming permeable to the reducing agent under the conditions of our experiment. In order to distinguish between these two possibilities $^{31}P$ NMR was carried out on sonicated PS/PESL vesicles (molar ratio 9:1). The $^{31}P$ NMR spectrum consisted of two peaks with the expected intensity ratio of about 9:1. The minor peak due to PESL was shifted downfield by 0.62 ppm (data not shown). Blank experiments and published data showed that in the presence of $\sim 10^{-4}$ M $MnCl_2$ the $^{31}P$ NMR resonances originating from the external surface of the bilayer are broadened into the base line and hence cannot be detected. In the presence of $10^{-4}$ M $Mn^{2+}$, the $^{31}P$ signal from PESL almost vanished, indicating that most of the spin-label PESL is located in the outer monolayer readily accessible to $Mn^{2+}$ (data not shown). The $^{31}P$ NMR experiment supports the notion that PESL is asymmetrically distributed in PS bilayers of small unilamellar vesicles, most of the spin-label being present in the outer monolayer.

The rate of reduction by photogenerated $FADH_2$ in the second slow phase depended on a number of parameters, e.g., the nature of the phospholipid and the spin probe (cf. Table I and Figure 5), the temperature (Figure 6), the FAD concentration, and hence the EDTA/FAD molar ratio (Figure 7). The rate of reduction was 6–20 times faster with bilayers labeled with CAT 16 than with bilayers containing PESL (Table I). As with the ascorbate method, the rate of reduction by $FADH_2$ increased with temperature. However, from an inspection of Figures 5 and 6 it is clear that reliable values for the fraction of internal phospholipid can only be derived from the time course of the reduction by extrapolation to zero time. The value for internal phospholipid thus derived for EPC/PESL vesicles at 1 and 21 °C was 33% (Table I, Figures 5 and 6). At temperatures of ≥30 °C this fraction appeared to increase, and at 37 °C it was more than 45% as shown in Figure 6. It could be shown by gel filtration on Sepharose 4B that this increase at temperatures of ≥30 °C was due to vesicle fusion leading to the formation of large unilamellar vesicles and possibly multilamellar structures.

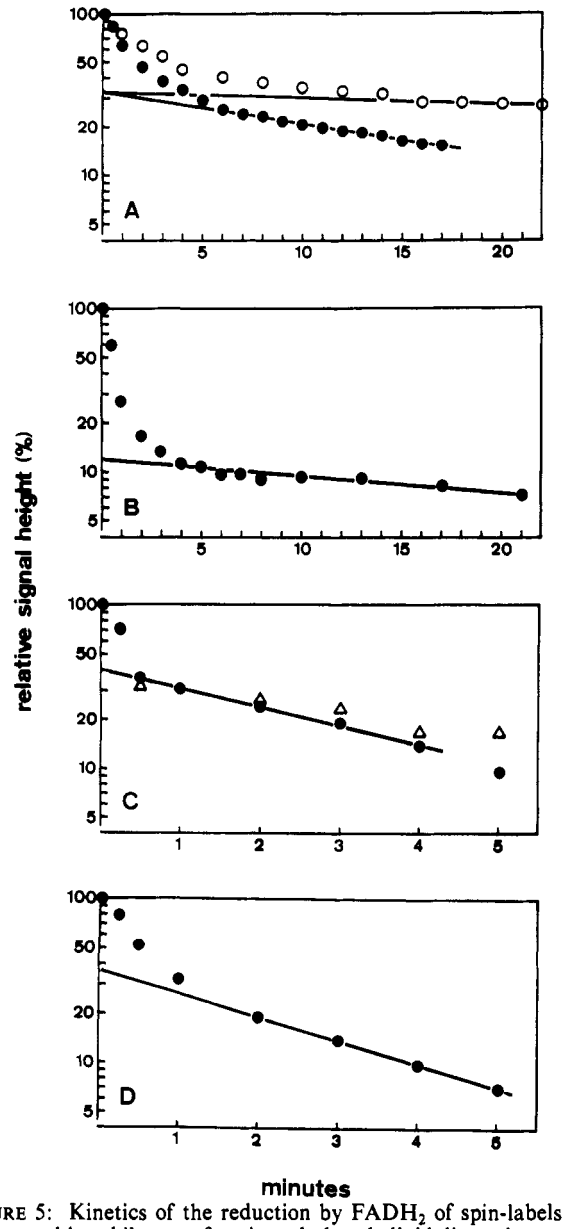


FIGURE 5: Kinetics of the reduction by $FADH_2$ of spin-labels incorporated into bilayers of sonicated phospholipid dispersions: (A) EPC labeled with PESL at 1 °C (○) and 21 °C (●); (B) PS labeled with PESL at 21 °C; (C) EPC labeled with CAT 16 at 21 °C; (D) PS labeled with CAT 16 at 21 °C. Sonicated, spin-labeled phospholipid dispersions were prepared as described under Materials and Methods. The photogeneration of $FADH_2$ from FAD with EDTA was accomplished under anaerobic conditions by exposing the sample to white light as described under Materials and Methods. Signal heights were normalized and were plotted as a function of the reaction time, i.e., the time the phospholipid dispersion was exposed to white light in the presence of EDTA/FAD. The following concentrations were used: (A) [EPC] = 31 mM, [FAD] = 0.063 mM, [EDTA] = 12.5 mM; (B) [PS] = 38 mM, [FAD] = 1.09 mM, [EDTA] = 18.8 mM; (C) [EPC] = 32 mM, [FAD] = 0.031 mM, [EDTA] = 12.5 mM (●) and [EPC] = 43 mM, [FAD] = 0.031 mM, [EDTA] = 313 mM (△); (D) [PS] = 13 mM, [FAD] = 0.022 mM, [EDTA] = 11.8 mM. The [phospholipid]/[spin-label] ratio was 100.

Figure 7 demonstrates that the kinetics of the second slow phase of the reduction by $FADH_2$ depends on the $FADH_2$ concentration: the rate of reduction decreased as the FAD concentration was reduced or as the [EDTA]/[FAD] increased. Optimum conditions for the reduction of spin-labels by photogenerated $FADH_2$ are (I) low temperatures (~0 °C) and (II) substoichiometric concentrations of FAD. Under these conditions the reduction of spin-label in the second slow phase is minimal. Therefore, reasonable estimates of the

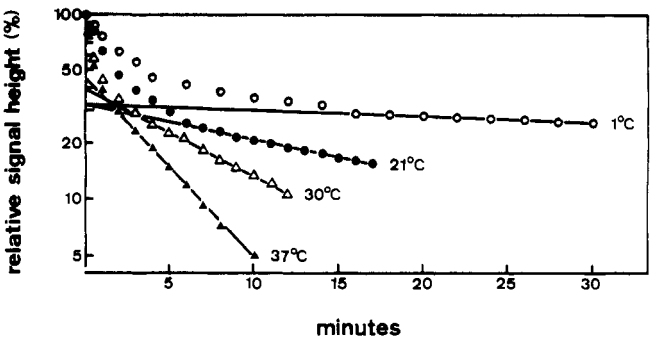


FIGURE 6: Temperature dependence of the reduction of PESL incorporated into bilayers of unilamellar EPC vesicles, with $FADH_2$. Sonicated, spin-labeled phospholipid dispersions were made as described under Materials and Methods. Light-mediated reduction of FAD was carried out in the presence of EDTA (12.5 mM) by exposing the sample to white light as described under Materials and Methods. Signal heights of the center line of the ESR spectrum were normalized and plotted as a function of the reaction time, i.e., the time the phospholipid dispersion was exposed to white light in the presence of [FAD] = 0.063 mM and [EDTA] = 12.5 mM, [EDTA]/[FAD] = 200. Temperatures at which the reduction was carried out are given on the right of each plot.

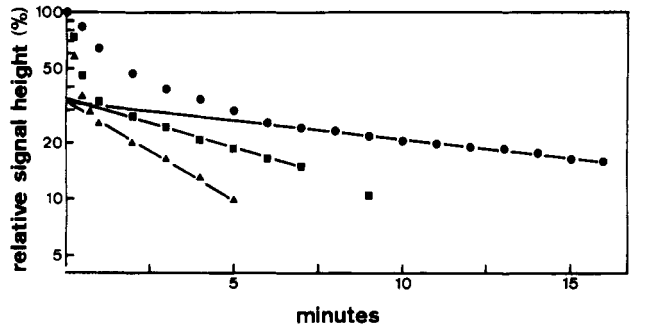


FIGURE 7: Kinetics of the reduction of PESL incorporated into bilayers of sonicated EPC dispersions (32 mM) in buffer at 21 °C. The spin-label was reduced by photogenerated $FADH_2$ in the presence of excess EDTA (12.5 mM). The FAD concentrations and [EDTA]/[FAD] were as follows: [FAD] = 0.063 mM, [EDTA]/[FAD] = 200 (●); [FAD] = 0.31 mM, [EDTA]/[FAD] = 40 (■); [FAD] = 1.25 mM, [EDTA]/[FAD] = 10 (▲).

fraction of internal phospholipid can be obtained from ESR measurements with photogenerated $FADH_2$ after 10–15 min (see Figures 5 and 6). In this case the tedious extrapolation to zero time was avoided.

*Determination of Internal Vesicle Volume.* Small unilamellar vesicles of EPC containing 1.0–1.3 mM Tempocholine in the dispersion medium as well as in the internal cavity of the vesicles were produced as described under Materials and Methods. When sodium ascorbate (25 mM) was added to such an EPC dispersion (23 mg/mL = 30 mM; 1.0 mM Tempocholine), Tempocholine present in the external medium was reduced in a fast reaction. Similarly, external Tempocholine was also reduced by adding a mixture of EDTA (12.5 mM) and FAD (0.016 mM) and exposing the sonicated EPC dispersion (29 mg/mL = 38 mM; 1.3 mM Tempocholine) to white light (see Materials and Methods). In the second slow phase of the reduction, Tempocholine present in the internal vesicle cavity reacted with the reducing agents. The values of the pseudo-first-order rate constants derived from the linear semilogarithmic plots (data not shown) were $k_1 = 2.7\ h^{-1}$ ($t_{1/2} = 0.26$ h) and $k_1 = 16.1\ h^{-1}$ ($t_{1/2} = 0.043$ h) for the reduction with sodium ascorbate and the reduction with photogenerated $FADH_2$ at 21 °C, respectively. As noted before (cf. Table I) the kinetics of the reduction with $FADH_2$ were faster than those of the ascorbate method. Extrapolation of the linear

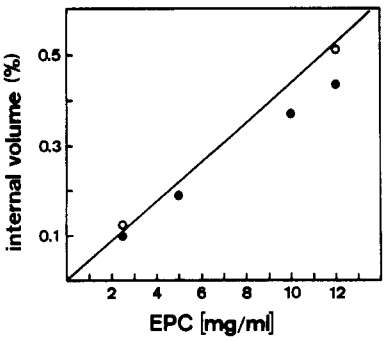


FIGURE 8: Internal volume (% of total solution) of sonicated EPC unilamellar vesicles as a function of EPC concentration (mg/mL). The internal volume, i.e., the volume of the vesicle cavity, was determined by the protective volume assay with 3 mM Tempocholine and 35 mM sodium ascorbate at 0 °C (○) and at room temperature (●) as described under Materials and Methods. The solid line represents the internal volume determined by entrapment of radioactive $^{22}NaCl$ as described by Hauser et al. (1973).

semilogarithmic plots to zero time yielded values for the internal volume of the vesicle cavity. The values for sonicated EPC dispersions of 10 mg/mL were 0.37 ± 0.03% and 0.27 ± 0.07% derived from the ascorbate and the $FADH_2$ method, respectively. These values are in reasonably good agreement with each other and the expectation value of 0.33% calculated for EPC (10 mg/mL) with a vesicle diameter of 25 nm, a bilayer thickness of 5 nm, and a phospholipid density of about 1. With the ascorbate method, linear relations between internal vesicle volume and EPC concentration were obtained (Figure 8). The solid line in Figure 8 represents an independent determination of the internal vesicle volume based on the entrapment of radioactive $^{22}NaCl$. The sodium ascorbate method at 0 °C agrees well with the radiotracer technique.

## Discussion

Unilamellar lipid vesicles are used in food products, cosmetics, pharmaceuticals, and medicine [reviewed by Ostro (1987)]. Further industrial and medical applications are anticipated. In the light of this, routine methods for the characterization of lipid vesicles are highly desirable. The knowledge of the lipid distribution between the outer and inner monolayer of the bilayer and the entrapped volume are important if unilamellar vesicles are used for encapsulation purposes (Szoka, 1986).

The ESR methods discussed here are suitable for the routine determination of these parameters. The ascorbate method was originally worked out for unilamellar EPC vesicles labeled with a phosphatidylcholine spin-label having the Tempo group covalently bonded to the quaternary ammonium group of choline (Kornberg & McConnell, 1971). As shown here, the ascorbate method suffers from two serious disadvantages. First, it is not universally applicable, but restricted to certain bilayer systems and spin-labels. For instance, the method failed when applied to bilayers labeled with negatively charged spin probes. Second, as shown before, sodium ascorbate is not impermeable to lipid bilayers (Kornberg & McConnell, 1971). Consequently experiments have to be conducted at low temperatures (at about 0 °C), or alternatively, a tedious extrapolation procedure is required in order to obtain meaningful results.

The purpose of this study is to improve the existing methodology. The new method described here is based on photogenerated $FADH_2$ as the reducing agent. $FADH_2$ is produced in situ from its oxidized form FAD by photolysis in the presence of excess EDTA as the reducing agent (Massey et al., 1978). The mechanism of this photoreduction according to eq 2 has been investigated by Massey et al. (1978) and Traber et al. (1982). Exposing the EDTA/FAD mixture to white light under anaerobic conditions induces the reduction of spin-label by photogenerated $FADH_2$. In a series of blank experiments, we were able to show that a mixture of EDTA/FAD is essential. FAD need not be present in excess or even stoichiometric quantities with respect to the spin-label; on the contrary, the best results are obtained with substoichiometric quantities of FAD, e.g., [FAD] = 0.063 mM, [EDTA]/[FAD] = 200, and [spin-label] $\simeq$ 0.3 mM. Exposure of our spin-labeled dispersions to light for 10 min in the presence or absence of EDTA has no effect on the ESR signal intensity: we conclude that EDTA per se does not reduce spin-labels and the spin-labels used are stable when exposed to white light at least up to 10 min. On the other hand, if the spin-labeled phospholipid dispersions are exposed to white light in the presence of FAD alone, i.e., without EDTA, the reduction of the spin-labels is surprisingly fast and follows monophasic kinetics. It has been shown (Knappe, 1975; Müller et al., 1988) that photolysis of FAD in the absence of reducing agents such as EDTA produces lipophilic flavin derivatives that presumably not only reduce spin-labels but also readily diffuse across the lipid bilayer. The resulting monophasic kinetics do not give information concerning the distribution of spin-label between inner and outer monolayers of the bilayer. Monophasic kinetics are also observed if sodium oxalate is substituted for EDTA in the photoreduction according to eq 2.

From the work presented here it is clear that the method based on the reduction with photogenerated $FADH_2$ is of general applicability. In contrast to water-soluble sodium ascorbate and the surface active, detergent-like L-ascorbic acid 6-palmitate, it works with both neutral and negatively charged bilayers that are spin-labeled with either positively or negatively charged probe molecules. It can be shown that it also works with bilayers labeled with uncharged spin probes, e.g., EPC bilayers labeled with neutral spin-labels such as 3-doxyl-5$\alpha$-cholestane (data not shown). For all spin-labeled bilayer vesicles, biphasic behavior is observed (Figure 5). This indicates that the spin-label present in bilayers of small unilamellar vesicles is distributed between two pools differing in their accessibility to the reducing agent. In the presence of excess amounts of detergents such as 23 mM sodium cholate known to solubilize phospholipid bilayers to small mixed micelles, only one pool of spin-label is observed. Under these conditions monophasic kinetics prevail with rate constants similar to those obtained in the first fast phase of the reduction. On the basis of these observations and reports in the literature (Kornberg & McConnell, 1971), we assign the readily accessible pool to spin-label located in the outer monolayer of the bilayer and the less readily available pool to spin-label present in the inner monolayer. The pseudo-first-order constants $k_1$ for the fast reduction of PESL or CAT 16 present in the outer layer of the bilayer of different phospholipid dispersions are included for comparison in Table I. The $k_1$ values for the reduction by photogenerated $FADH_2$ at 21 °C vary between $\sim$25 and 125 $h^{-1}$ under the conditions of our experiments; the $k_1$ values for the reduction of CAT 16 by ascorbate are too fast to be measurable. The second slow phase of reduction is then due to spin-label undergoing "flip-flop" motion or to permeation of the reducing agent through the bilayer or a combination of these two processes.

Both the ascorbate and the $FADH_2$ method behave similarly in the protective volume assay. Under physiological conditions

both methods give useful approximations for the entrapped vesicle volume and are therefore suitable for the routine determination of this parameter.

The relatively fast kinetics of the slow-phase reduction with photogenerated $FADH_2$ are unexpected. EDTA and FAD are fairly large molecules bearing two negative charges each under the conditions of our experiments. Therefore, they are expected to be impermeable to lipid bilayers. However, the rate of reduction observed in the second slow phase corresponding to the reduction of internal spin-label is surprisingly high (cf. Figures 5–7 and Table I). As mentioned above, this is probably due to the photogeneration of side products of FAD. Attempts to suppress this side reaction by an excess of EDTA were only partially successful. Figure 5C shows that EDTA concentrations of ~300 mM ([EDTA]/[FAD] $\approx 10^4$) causes only a 30% decrease of the rate of reduction in the slow phase compared to standard EDTA concentrations of 12.5 mM.

More work, particularly on the reaction mechanism, is required in order to explain the relatively fast kinetics of the reduction of internal spin-label. This will be the subject of an independent study. We note that the fast kinetics in the second phase of the reduction is one disadvantage of the photoreduction method. In order to obtain precise information on membrane asymmetry and entrapped vesicle volume, the recording of the time course of the reduction and extrapolation of the data to zero time are necessary.

## ACKNOWLEDGMENTS

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**Registry No.** $FADH_2$, 1910-41-4; EDTA, 60-00-4; FAD, 146-14-5; DPPE, 5681-36-7; CAT 16, 114199-16-5; PESL, 118111-50-5; succinimidyl 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylate, 2154-67-8.

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