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Kinetics of Cholesterol and Phospholipid Exchange from Membranes Containing Cross-Linked Proteins or Cross-Linked Phosphatidylethanolamines†

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ABSTRACT: Mono- and dipalmitoylphosphatidylethanolamine derivatives have been synthesized and used to evaluate the role of cross-links between the amino groups of two phospholipid molecules in the rate of cholesterol movement between membranes. Incorporation of the cross-linked phospholipids into small unilamellar vesicles (the donor species) decreased the rate of spontaneous cholesterol exchange with acceptor membranes (small unilamellar vesicles or *Mycoplasma gallisepticum* cells). These results suggest that the cross-linking of aminophospholipids by reactive intermediates, which may be one of the degenerative transformations associated with peroxidation of unsaturated lipids and cellular aging, can inhibit cholesterol exchangeability in biological membranes. The rates of spontaneous [^{14}C]cholesterol and protein-mediated ^{14}C-labeled phospholipid exchange from diamide-treated mycoplasma and erythrocyte membranes have also been measured. The formation of extensive disulfide bonds in the membrane proteins of *M. gallisepticum* enhanced the ^{14}C-labeled phospholipid exchange rate but did not affect the rate of [^{14}C]cholesterol exchange. The rates of radiolabeled cholesterol and phospholipid exchange between erythrocyte ghosts and vesicles were both enhanced (but to different extents) when ghosts were treated with diamide. These observations suggest that diamide-induced oxidative cross-linking of sulfhydryl groups in membrane proteins does not lead to random defects in the lipid domain.

Lipid peroxidation and protein cross-linking are two factors that influence the rates of lipid translocation in membranes. Peroxidation of unsaturated fatty acyl chains alters the physical properties of the lipids and can also cause covalent modifications in proteins and lipids by reactions involving reactive intermediates. If critical levels of peroxidation products are present, the rates of lipid transbilayer movement are stimulated (Shaw & Thompson, 1982). It has been proposed that nonbilayer lipid structures are produced from cross-linking of membrane lipids (Barsukov et al., 1980). Cholesterol was found to reduce the rate of lipid movement in some membranes exposed to peroxidation, possibly by stabilizing the bilayer (Shaw & Thompson, 1982) or suppressing the extent of peroxidation (Mowri et al., 1984). Malondialdehyde, which is formed during peroxidation of unsaturated lipids, can cross-link a variety of biological macromolecules, including the amino groups of phospholipids and proteins through Schiff base formation (Jain & Hochstein, 1980; Rice-Evans & Hochstein, 1981; Jain & Shohet, 1982). There has been considerable interest in the effects of malondialdehyde on biological systems since it has been suggested that cross-linking of macromolecules contributes to cellular aging and other degenerative processes [e.g., Bland (1978)]. Although the structure of the product from the reaction of phosphatidylethanolamine (PE)[1] with malondialdehyde has not been established (Bidlack & Tappel, 1973; Shimasaki et al., 1984),

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[1] Abbreviations: FNPS, bis(4-fluoro-3-nitrophenyl) sulfone; PBS, 150 mM NaCl, 5 mM KCl, 5.5 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, and 0.5 mM $CaCl_2$, pH 7.4; PDIT, 1,4-phenylene diisothiocyanate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; nitrosulfone-mono-PE, 4-(α,β-dipalmitoylphosphatidylethanolamino)-3,3′-dinitro-4′-hydroxydiphenyl sulfone; nitrosulfone-di-PE, bis[4-(α,β-dipalmitoylphosphatidylethanolamino)-3-nitrophenyl] sulfone; α,β- or α,γ-PDIT-mono-PE, *N*-[4-[(α,β- or α,γ-dipalmitoylphosphatidylethanolamino)thioformamido]phenyl]thiourea; α,β- or α,γ-PDIT-di-PE, *N*,*N*′-1,4-phenylenebis[(α,β- or α,γ-dipalmitoylphosphatidylethanolamino)thioformamide]; 1,4-xylylene-di-PE, *N*,*N*′-1,4-xylylenebis(α,β-dipalmitoylphosphatidylethanolamine); STM, 0.40 M sucrose, 50 mM tris(hydroxymethyl)aminomethane, and 20 mM $MgCl_2$, pH 7.4; SUV, small unilamellar vesicles; SDS, sodium dodecyl sulfate.

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a 1:1 adduct was identified recently in which the amino groups of PE and phosphatidylserine appear to be cross-linked (Jain & Shohet, 1984). Prior to the discovery of such adducts, we sought to test the possibility that cross-linking of aminophospholipids can regulate the spontaneous exchange of cholesterol between membranes (Robinson et al., 1983). Three cross-linked DPPE derivatives have been synthesized and incorporated into SUV prepared from PC and radiolabeled cholesterol. Since the coupling agent used to effect the cross-linking may also influence the [^{14}C]cholesterol kinetics, we also measured the rates of exchange in SUV containing monomeric DPPE derivatives that are covalently attached to the coupling agent.

The mild oxidizing agent diamide causes extensive cross-linking of intra- and intermolecular sulfhydryl groups of spectrin and other proteins in erythrocyte membranes, leading to weakening of direct interactions between the membrane skeleton and negatively charged phospholipids (Haest et al., 1978; Mohandas et al., 1982). The phospholipids that are normally localized preferentially in the inner leaflet of the erythrocyte membrane become redistributed, and the rates of movement of several phospholipids are enhanced by extensive disulfide bond formation (Haest et al., 1978; Franck et al., 1982; Mohandas et al., 1982). In the present paper, we have examined the rates of [^{14}C]cholesterol and ^{14}C-labeled phospholipid exchange from diamide-treated mycoplasma and erythrocyte membranes. Although the mechanisms of cholesterol and phospholipid translocation in these membranes are not known, our results suggest that the diamide-induced structural defects in the lipid domain (Deuticke et al., 1983) do not represent a general disturbance of the bulk lipids.

Materials and Methods

Materials. α,β- and α,γ-DPPE, egg PC, oleic and palmitic acids, FNPS, dicetyl phosphate, cholesterol (recrystallized twice from ethanol), diamide, bovine serum albumin (fraction V, fatty acid poor), and deoxyribonuclease were purchased from Sigma. The sources of [4-^{14}C]cholesterol, [1-^{14}C]palmitic acid, algae [U-^{14}C]PC, and [2-^{3}H]glycerol trioleate were as cited previously (Clejan & Bittman, 1984). DEAE-Sepharose CL-6B was obtained from Pharmacia and Sigma. Terephthalaldehyde was from Aldrich, and PDIT was from Eastman. Triethylamine was distilled and dried over CaH_2. Isopropyl alcohol was dried over molecular sieves (3 Å). The purities of the lipids were checked by thin-layer chromatography as described previously (Clejan et al., 1981).

Preparation of SUV. Aqueous dispersions of the donor and acceptor species were prepared as described previously (Clejan & Bittman, 1984) and sonicated under nitrogen in a Heat Systems W375A sonicator equipped with a cup horn or microtip. Undispersed lipid and multilamellar vesicles were removed by centrifuging the dispersions for 2 h at 40000 rpm in a Beckman Type 50 rotor.

Growth of Mycoplasma Cells. M. gallisepticum strain A5969 was grown in a modified Edward medium containing cholesterol (2 or 10 μg/mL), palmitic and oleic acids (10 μg of each/mL), and albumin (1% w/v) as described (Clejan et al., 1981; Clejan & Bittman, 1984).

Preparation of Labeled Ghosts. Ghosts were prepared from recently outdated human erythrocytes as described previously (Bittman et al., 1984). After the last centrifugation, the hemoglobin-free pellet was suspended in PBS buffer. For labeling with [^{14}C]cholesterol, the ghosts were incubated for 18 h with gentle shaking with a 15-fold excess of cholesterol/egg PC SUV (molar ratio 0.95) containing 10 μCi/mL [^{14}C]cholesterol. The incubation medium contained PBS buffer supplemented with penicillin (1000 units/mL) and albumin (1% w/v). For labeling with [^{14}C]PC, the ghosts were incubated with SUV containing 12 μCi/mL [^{14}C]PC, and the incubation medium included 300 μg/mL nonspecific exchange protein. The latter was isolated from human plasma by a procedure similar to that of Tall et al. (1983); the phenyl-Sepharose and carboxymethylcellulose steps were included, but chromatography on heparin–Sepharose was omitted. The specific activity of the preparation was estimated as 28 nmol of PC transferred from SUV to multilamellar liposomes per milligram of exchange protein per minute at 37 °C, by using the assay system of Crain & Zilversmit (1980). At the end of the incubation period, the ghosts were collected by centrifugation in an Eppendorf centrifuge for 10 min, washed 3 times with PBS buffer, and divided into 0.5-mL aliquots that were stored at −70 °C.

[^{14}C]Cholesterol and ^{14}C-Labeled Phospholipid Exchange. The kinetic measurements were carried out at 37 °C. When mycoplasma cells or membranes containing radiolabeled cholesterol or phospholipid were used as the donor species (Table II), the incubation medium contained the cells (about 0.5 mg of cell protein/mL) or membranes (about 0.2 mg of membrane protein/mL) and SUV (1.18 μmol/mL of total lipid, which corresponds to an ~20-fold excess of acceptor species) in STM buffer containing penicillin G (5000 units/mL), deoxyribonuclease (20 μg/mL), albumin (2% w/v), and dimethyl sulfoxide (3% v/v). Aliquots (0.5 mL) were withdrawn in duplicates at time intervals, and cells were pelleted by centrifugation for 2 min as described previously (Clejan & Bittman, 1984). Isolated membranes of *M. gallisepticum* were pelleted quantitatively by centrifugation in a Beckman Airfuge at 20 psi for 10 min at room temperature. The acceptor SUV contained [^{3}H]glycerol trioleate as a nonexchangeable marker; the pellet obtained after centrifugation contained <5% of the radioactivity of the SUV. The supernatant contained <2% of the ^{14}C counts per minute (cpm) of the donor cell membranes, demonstrating that the rapid centrifugation procedure separated the donor and acceptor species. The ^{3}H cpm found in the pellet did not increase significantly during incubation periods of 12 h, indicating that sticking of SUV to the mycoplasma membrane was negligible under these conditions. The calculations of the fraction of radiolabeled lipid exchanged and of the half-time were described previously (Clejan & Bittman, 1984). When SUV containing PE derivatives, egg PC, dicetyl phosphate, and [^{14}C]cholesterol were used as the donor species (Table I and Figure 3), the negatively charged SUV were separated from the excess of uncharged acceptor SUV on short columns of DEAE-Sepharose CL-6B (McLean & Phillips, 1981). The SUV were prepared in 20 mM sodium phosphate buffer, pH 6.0, and the recovery of the acceptor SUV in the eluate was about 85–90% on the basis of the cpm of [^{3}H]glycerol trioleate. The extent of fusion was estimated after a 12-h incubation period by using donor SUV containing [^{14}C]cholesteryl oleate and acceptor SUV containing [^{3}H]glycerol trioleate. From the ^{14}C cpm in the eluate, it was estimated that <6% fusion took place. In order to examine whether [^{14}C]cholesterol underwent exchange without chemical modification, thin-layer chromatography of the lipids extracted from the acceptor SUV was performed; ~97% of the [^{14}C]cholesterol cochromatographed with a cholesterol standard. When SUV were used as the donor species and mycoplasma cells as the acceptor (Table I and Figure 3), the incubation was conducted in STM buffer containing penicillin, deoxyribonuclease, and albumin (1% w/v), with a 20-fold excess of acceptor with respect to

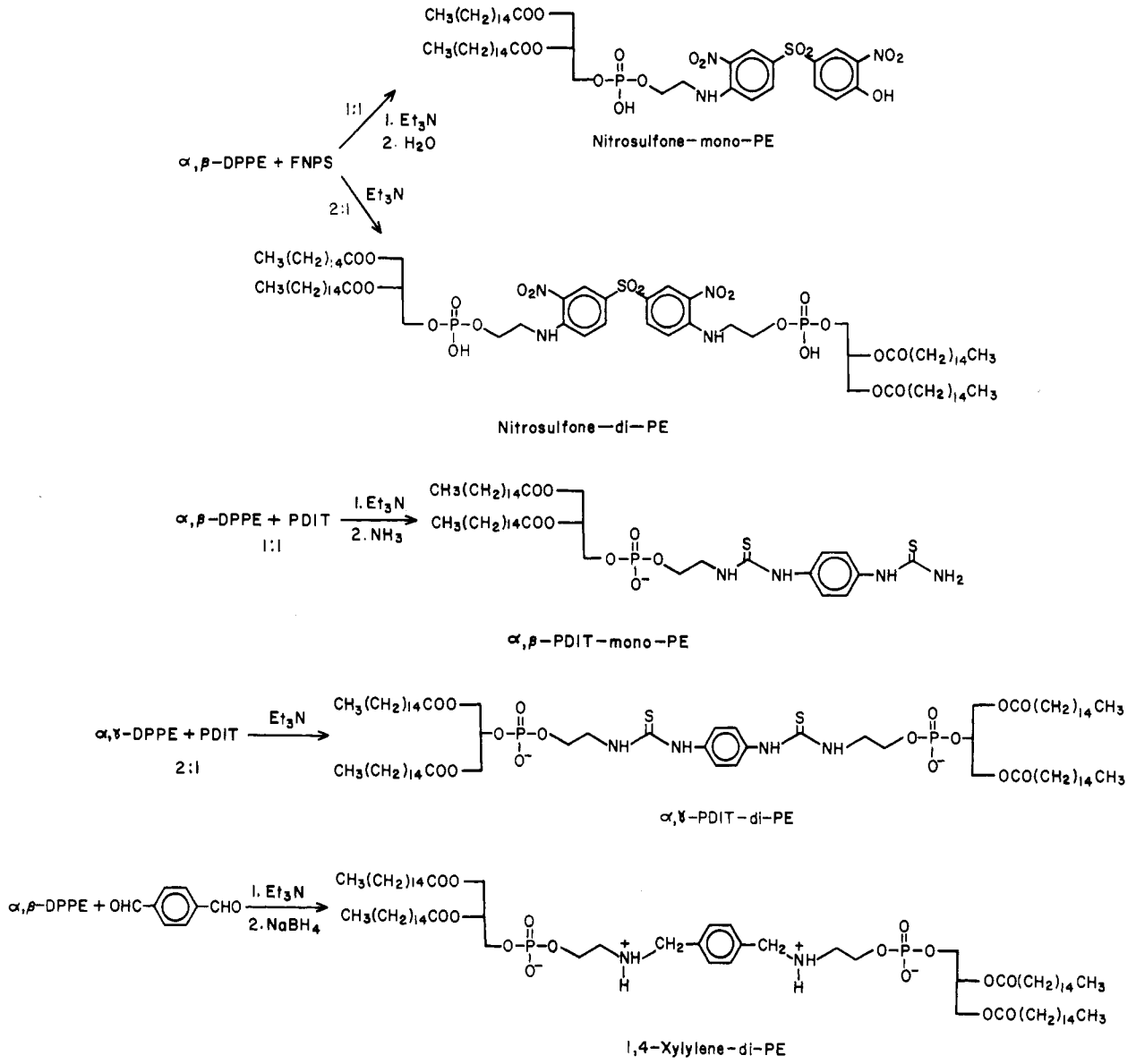

FIGURE 1: Structures of PE derivatives and reactions by which they were prepared. The mono-PE derivatives are the mono-PE nitrosulfone [4-(α,β-dipalmitoylphosphatidylethanolamino)-3,3′-dinitro-4′-hydroxydiphenyl sulfone], the mono-PE α,γ-PDIT [*N*-[4-[(α,γ-dipalmitoylphosphatidylethanolamino)thioformamido]phenyl]thiourea], and the corresponding mono-PE α,β-PDIT (not shown). The di-PE derivatives are the di-PE nitrosulfone [bis[4-(α,β-dipalmitoylphosphatidylethanolamino)-3-nitrophenyl] sulfone] and the di-PE xylylene [*N*,*N*′-1,4-xylylenebis(α,β-dipalmitoylphosphatidylethanolamine)].

lipid concentration. The fraction of [^{14}C]cholesterol exchanged into the mycoplasma cells was calculated from the cpm in the cell pellet (Clejan & Bittman, 1984). The infinity value was determined after 20–24 h. When erythrocyte ghosts were used as the donors of [^{14}C]cholesterol or [^{14}C]PC (Figures 4 and 5), the acceptor SUV (containing the same cholesterol/phospholipid molar ratio as the donor) were present in 15-fold excess. The incubation was conducted in PBS buffer containing penicillin G (1000 units/mL) and albumin (1% w/v). At various time intervals, 0.3-mL aliquots were withdrawn in duplicates, and the ghosts were pelleted by centrifugation for 10 min in an Eppendorf centrifuge. The ghosts were washed once in PBS buffer and recentrifuged. The supernatant was removed by aspiration, and the ghost pellet was transferred to scintillation vials and counted as described for the mycoplasma cell pellet (Clejan & Bittman, 1984). Only a trace amount (<2%) of [^{3}H]glycerol trioleate from the acceptor SUV was found in the ghosts.

Analytical Procedures. The methods for extraction of lipids from *Mycoplasma* cell suspensions and for determination of protein, phospholipid, and cholesterol were described previously (Clejan et al., 1981). Lipids were extracted from erythrocyte ghosts with isopropyl alcohol/chloroform (Rose & Oklander, 1965). The amount of nitrosulfone-mono-PE or nitrosulfone-di-PE incorporated into SUV or mycoplasma membranes was determined from the absorbance at 390 nm of the extracted lipids, followed by comparison with a standard curve. The extent of cross-linked proteins produced by treating mycoplasma cells on membranes or ghosts with diamide was assessed by sodium dodecyl sulfate (1%) electrophoresis on 5% polyacrylamide gels containing 0.1% *N*,*N*′-methylenebis(acrylamide). The samples were solubilized by heating 9 volumes of packed cells or membranes (2 mg of protein) with 0.1 volume of 20% SDS at 100 °C for 2 min. After electrophoresis, the gels were stained with Coomassie blue.

Synthesis of Cross-Linked PE Derivatives. The reactions we used to synthesize the monomeric and dimeric PE derivatives are outlined in Figure 1. Each di-α,β-DPPE derivative was prepared as a mixture of diastereomers, whereas the mono- and di-α,γ-DPPE derivatives and the mono-α,β-DPPE de-

rivative were optically inactive.

Nitrosulfone-mono-PE. A mixture of DL-α,β-DPPE (100.6 mg, 0.145 mmol), FNPS (55.3 mg, 0.161 mmol), and triethylamine (43 μL, 0.31 mmol) in 1.5 mL of alcohol-free $CHCl_3$ was stirred for 20 h at room temperature. Concentrated aqueous ammonium hydroxide (14 μL) was added, and stirring was continued for 30 min. On addition of 0.6 mL of MeOH the reaction mixture became homogeneous. The reaction mixture was spotted onto two silica gel GF thin-layer plates (1 mm thick, Analtech, Newark, DE), which were then dried for 30 min at room temperature (2 torr) and eluted with $CHCl_3$/MeOH/28% NH_4OH (80/20/2 v/v/v). Two major yellow bands were observed. The slower moving band (R_f = 0.28) was identical in R_f with the spot identified as nitrosulfone-di-PE (see below). The faster moving band (R_f = 0.58) was scraped from the plate and eluted once with 25 mL of $CHCl_3$/MeOH (4/1 v/v) and 3 times with 15 mL of $CHCl_3$/MeOH (1/1 v/v). The solvents were removed, and the residue was dissolved in 25 mL of $CHCl_3$ and washed with two 15-mL portions of 0.5 N HCl and 30 mL of water. The chloroform solution was dried with Na_2SO_4 and evaporated in vacuo. The resultant residue was dissolved in $CHCl_3$ (1 mL), filtered, and added, with stirring, to 20 mL of acetonitrile to which 1 drop of 88% formic acid had been added. The turbid mixture was cooled at −20 °C for 1 h, producing a precipitate that was collected by centrifugation at −20 °C and washed with acetonitrile and a small amount of MeOH at −20 °C. The solvents were removed in vacuo to yield 68 mg (46%) of nitrosulfone-mono-PE as a light yellow powder. Anal. Calcd for $C_{49}H_{80}N_3O_{15}PS$: C, 58.03; H, 7.95; N, 4.14; P, 3.05; S, 3.16. Found: C, 56.98; H, 7.87; N, 4.17; P, 2.98; S, 3.25.

Nitrosulfone-di-PE. A mixture of DL-α,β-DPPE (199 mg, 0.288 mmol), FNPS (50 mg, 0.145 mmol), and triethylamine (85 μL, 0.61 mmol) was stirred in 3 mL of alcohol-free $CHCl_3$ for 20 h at room temperature. The reaction mixture was spotted onto two silica gel GF plates (2 mm thick), which were then dried for 30 min at room temperature (2 torr) and eluted with $CHCl_3$/MeOH/28% NH_4OH (85/15/2 v/v/v). The major band (R_f = 0.28) was scraped from the plate and eluted with 50 mL of 4/1 (v/v) $CHCl_3$/MeOH. The solvents were removed in vacuo, and the residue was dissolved in $CHCl_3$ (25 mL). The $CHCl_3$ solution was washed with 0.5 N HCl (2 × 20 mL) and water (1 × 10 mL) and then dried with Na_2SO_4. Evaporation of $CHCl_3$ in vacuo afforded a residue, which was dissolved in 2 mL of chloroform, filtered, and added to 40 mL of vigorously stirring acetonitrile. The mixture was kept at 0 °C for 2 h, and the resulting yellow precipitate was collected by filtration and washed with cold acetonitrile to provide (after drying) 145 mg (58%) of nitrosulfone-di-PE as a light yellow powder. Anal. Calcd for $C_{86}H_{152}N_4O_{22}P_2S{\cdot}2H_2O$: C, 59.91; H, 9.12; N, 3.25; P, 3.59; S, 1.86. Found: C, 59.89; H, 9.04; N, 3.31; P, 3.51; S, 2.06.

α,β-PDIT-mono-PE. A mixture of DL-α,β-DPPE (152 mg, 0.220 mmol), PDIT (100 mg, 0.520 mmol), and triethylamine (40 μL, 0.29 mmol) was stirred in 3 mL of alcohol-free $CHCl_3$ for 2 days at 30 °C and then kept at −20 °C for 2 days. Concentrated ammonium hydroxide (80 μL) and MeOH (1 mL) were added, and the mixture was stirred for 2 h. The precipitate was discarded, and the product was isolated from the filtrate by preparative thin-layer chromatography as described for α,γ-PDIT-di-PE. A concentrated solution of α,β-PDIT-mono-PE in chloroform was precipitated from acetonitrile at −20 °C, yielding 97 mg (44%). Anal. Calcd for $C_{51}H_{96}N_5O_8PS_2{\cdot}2H_2O$: C, 58.99; H, 9.70; N, 6.74; S, 6.18. Found: C, 59.19; H, 9.68; N, 6.43; S, 6.79.

α,γ-PDIT-di-PE. A mixture of DL-α,γ-DPPE (199 mg, 0.288 mmol), PDIT (28 mg, 0.145 mmol), and triethylamine (50 μL, 0.36 mmol) was stirred at 30 °C for 46 h. The reaction mixture was spotted onto two silica gel plates (2 mm thick), which were dried (30 min, 2 torr) and eluted with $CHCl_3$/MeOH/28% NH_4OH (80/20/2 v/v/v). The major band was detected at 254 nm (R_f = 0.47), scraped from the plate, and eluted with 4/1 (v/v) $CHCl_3$/MeOH (1 × 50 mL) and 1/1 (v/v) $CHCl_3$/MeOH (5 × 15 mL). The solvents were removed in vacuo, and the residue was dissolved in 3 mL of $CHCl_3$ and then precipitated from 60 mL of acetonitrile at −20 °C. The precipitate was collected and dried, affording 165 mg (71%) of α,γ-PDIT-di-PE as a white amorphous powder. Anal. Calcd for $C_{82}H_{152}N_4O_{16}P_2S_2{\cdot}(C_2H_5)_3N{\cdot}2H_2O$ [analyzed as the mono(triethylammonium) salt dihydrate]: C, 61.69; H, 10.06; N, 4.09; P, 3.62; S, 3.74. Found: C, 60.40; H, 9.67; N, 3.95; P, 3.67; S, 3.37. α,γ-PDIT-mono-PE was prepared by washing a $CHCl_3$ solution of α,γ-PDIT-di-PE with 0.5 N HCl (2 × 20 mL) and water (1 × 10 mL); the residue was purified by preparative thin-layer chromatography using the solvent system cited above.

α,β-PDIT-di-PE. This compound was prepared in 52% yield from 200 mg of DL-α,β-DPPE as described for α,γ-PDIT-di-PE. Anal. Calcd for $C_{82}H_{152}N_4O_{16}P_2S_2{\cdot}2H_2O$: C, 61.09; H, 9.75; N, 3.48; S, 3.98. Found: C, 59.94; H, 9.72; N, 3.45; S, 4.26.

1,4-Xylylene-di-PE. To a mixture of DL-α,β-DPPE (201.5 mg, 0.291 mmol) and terephthalaldehyde (58 mg, 0.43 mmol) in 2 mL of alcohol-free $CHCl_3$ were added triethylamine (50 μL, 0.36 mmol) and molecular sieves (5 Å, 0.4 g). The reaction mixture dissolved on stirring for 4 h at room temperature, and the solution was allowed to stand at 5 °C for 2 days to complete the formation of the Schiff base. A suspension of sodium borohydride (20 mg, 0.5 mmol) in 2 mL of isopropyl alcohol was added dropwise, and the mixture was stirred at 5 °C overnight. After dilution with MeOH (1 mL) and $CHCl_3$ (1 mL), the mixture was filtered through Celite. The filtrate was spotted onto four silica gel GF plates (1 mm thick), which were then dried (30 min, 2 torr). After elution with $CHCl_3$/MeOH/28% NH_4OH (80/20/2 v/v/v), the major band was identified at 254 nm (R_f = 0.35), scraped from the plate, and eluted with 4/1 (v/v) $CHCl_3$/MeOH (1 × 50 mL) and 1/1 (v/v) $CHCl_3$/MeOH (4 × 20 mL). The solvents were removed in vacuo, and the residue (133 mg) was mixed with ammonium fluoride (200 mg), MeOH (5 mL), and $CHCl_3$ (5 mL). The mixture was stirred at room temperature for 2 h, diluted with $CHCl_3$ (35 mL), and washed 3 times with 25 mL of water. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. The residue (117 mg) was dissolved in 2 mL of $CHCl_3$ and then precipitated from 25 mL of acetonitrile at −20 °C. The precipitate was collected by centrifugation at −20 °C and dried under vacuum to yield 111 mg (51%). Anal. Calcd for $C_{82}H_{154}N_2O_{16}P_2$: C, 66.28; H, 10.45; N, 1.89; P, 4.17. Found: C, 64.93; H, 10.49; N, 1.86; P, 3.64.

Results

Malondialdehyde, an end product of the peroxidation of membrane fatty acyl chains, is presumably generated in the lipid phase. It can react with phospholipids and proteins in the immediate vicinity, or it can move toward and react with molecules at the lipid/water interface. Reaction of malondialdehyde at the α-amino group of amino acids to form 1:1 and 1:2 adducts is relatively rapid (Nair et al., 1981). Adducts can be formed from the reaction of bifunctional agents with phospholipid and protein amino groups (Jain & Hochstein, 1980; Rice-Evans & Hochstein, 1981; Valtersson & Dallner,

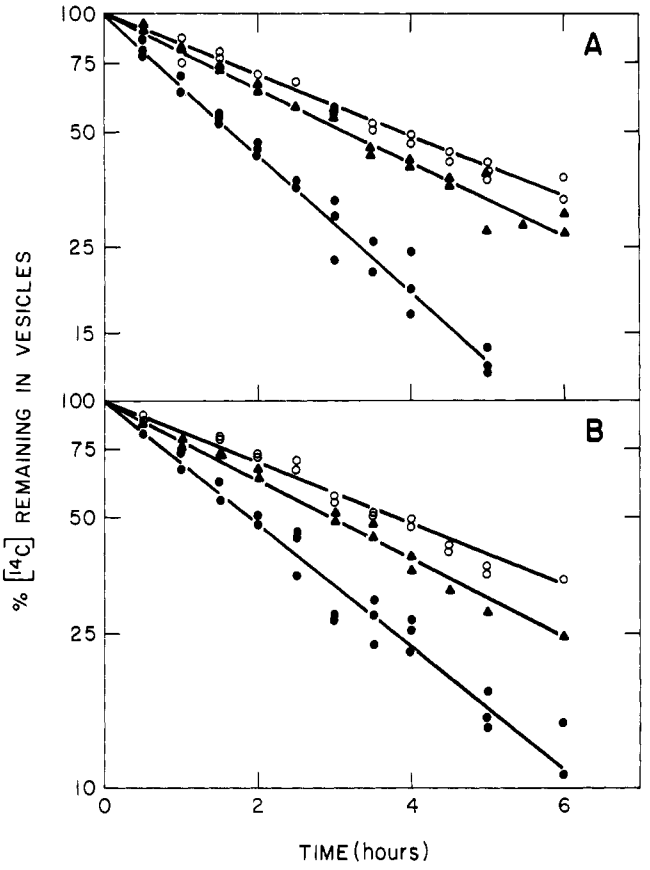

FIGURE 2: First-order kinetic plots of [^{14}C]cholesterol exchange from SUV containing mono- and bis(phosphatidylethanolamines) to egg PC/cholesterol SUV. The donor and acceptor SUV contained 25.5 mol % cholesterol. Acceptor SUV were present at a 12-fold excess. The negatively charged donor SUV were retained on DEAE-Sepharose CL-6B columns as described under Materials and Methods, whereas the uncharged acceptor SUV were eluted. The effects of incorporating the following PE derivatives are shown: (A) 1,4-xylylene-di-PE (○), nitrosulfone-di-PE (▲), and nitrosulfone-mono-PE (●); (B) α,β-PDIT-di-PE (○), α,γ-PDIT-di-PE (▲), and PDIT-mono-PE (●).

1982). We therefore prepared cross-linked PE derivatives in chloroform solution containing triethylamine, removing the possibility of cross-linking between phospholipids and proteins.

[^{14}C]Cholesterol Exchange between SUV. The effects of incorporating 25.4 mol % of the monomeric and dimeric PE derivatives into donor SUV are compared in Figure 2.[2] The first-order plots show that radiolabeled cholesterol is exchangeable in a single kinetic pool and that the exchange rates of cholesterol from SUV prepared with dimeric PE derivatives are lower than those from SUV containing the corresponding mono-PE analogues. Table I shows the half-times for [^{14}C]cholesterol exchange. Relative to the incorporation of nitrosulfone-mono-PE, the equivalent amount of nitrosulfone-di-PE caused a 100% decrease in the exchange rate. Similar decreases in rates were found when the PDIT-di-PE derivatives were incorporated and compared with the corresponding monomeric PE, which bears the coupling arm but lacks the second PE molecule. The rate of exchange from SUV containing α,γ-PDIT-di-PE was faster than that from SUV containing α,β-PDIT-di-PE. This suggests that nearest-neighbor interactions between cholesterol and phospholipids depend on the relative positions of the fatty acyl chains on the glycerol backbone. The increased separation between the two

[2] Each of the mono-PE derivatives increased the rate of [^{14}C]cholesterol exchange when incorporated into donor SUV, relative to donor species with the equivalent content of cholesterol and dicetyl phosphate.

Table I: Effect of Cross-Linking of Phosphatidylethanolamines on Half-Times for [^{14}C]Cholesterol Exchange

PE derivative incorporated into donor SUV[b]	$t_{1/2}$ (h) for exchange system[a]	
	SUV/SUV (donor/acceptor)	SUV/mycoplasma cells (donor/acceptor)
nitrosulfone-mono-PE	1.7 ± 0.2	3.5 ± 0.2
nitrosulfone-di-PE	3.3 ± 0.3	4.2 ± 0.3
α,γ-PDIT-mono-PE	1.9 ± 0.2	1.3 ± 0.1
α,γ-PDIT-di-PE	3.0 ± 0.2	2.2 ± 0.2
α,β-PDIT-di-PE	3.8 ± 0.2	
1,4-xylylene-di-PE	3.8 ± 0.2	

[a] The values are the average ± SD of two to four experiments with separate donor and acceptor preparations (each performed in duplicate). Donor SUV contained egg PC (36.5 mol %), the mono- or di-PE indicated (25.4 mol %), cholesterol (25.5 mol %), dicetyl phosphate (13.5 mol %), and a trace of [^{14}C]cholesterol (1 μCi). Acceptor SUV contained egg PC (74.5 mol %), cholesterol (25.5 mol %), and a trace of [^{3}H]glycerol trioleate (0.5 μCi). The total lipid concentrations of donor and acceptor SUV were 1.18 and 14.2 mM, respectively, and the relative volumes used of donor to acceptor SUV were 1:2. Acceptor *M. gallisepticum* cells had a cholesterol/phospholipid molar ratio of 0.25 (total lipid concentration of about 10.5 mM). [b] See Figure 1 for the structures of the PE derivatives.

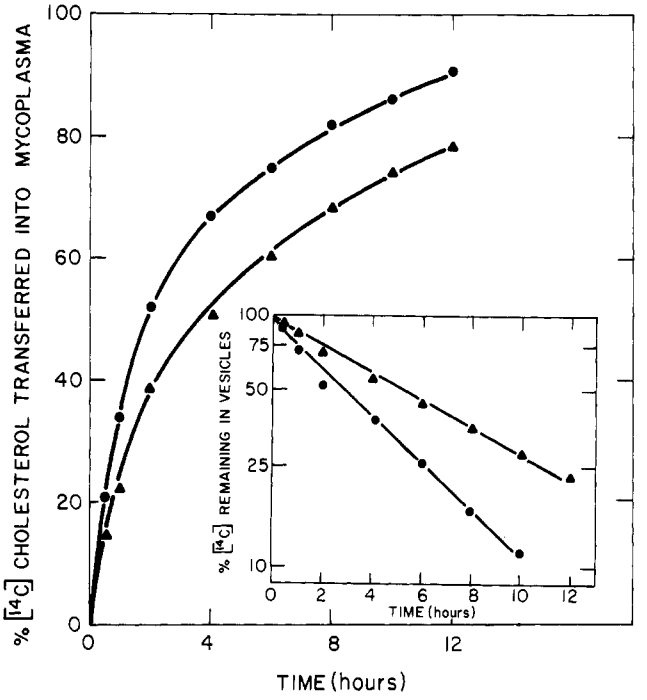

FIGURE 3: Rates of [^{14}C]cholesterol exchange from SUV containing PE derivatives to *M. gallisepticum* cells: PDIT-mono-PE (●); PDIT di-PE (▲). The incubation medium contained albumin (2% w/v). Cells were present at an excess of 20-fold relative to the lipid concentration of the donor SUV and were pelleted in an Eppendorf centrifuge as described under Materials and Methods. The inset is the first-order plot obtained for [^{14}C]cholesterol remaining in the donor SUV as a function of time of incubation with *M. gallisepticum* cells.

acyl chains has been reported to result in an increase in chain mobility in bilayers from α,γ-diacylphospholipids compared with bilayers from the analogous α,β isomers (Seelig et al., 1980; Smith, 1981).

[^{14}C]Cholesterol Exchange from SUV to Mycoplasma Cells. When *M. gallisepticum* cells were used as the acceptors of labeled cholesterol from SUV containing PE derivatives, the radioactivity appearing in the cell pellet was measured as a function of time. Figure 3 shows that the presence of PDIT-di-PE in the donor SUV suppressed the rate of cholesterol movement to mycoplasma cells, compared with donor SUV containing PDIT-mono-PE. The inset presents semilogarithmic plots of the percent of the total [^{14}C]cholesterol

Table II: Effect of Diamide Treatment of Mycoplasma Cell Membranes on the Half-Times for Spontaneous [^{14}C]Cholesterol and ^{14}C-Labeled Phospholipid Exchange between Cells or Membranes and SUV

lipid exchanged	treatment	$t_{1/2}$ (h) for exchange system[a] cells/SUV (donor/acceptor)	membranes/SUV (donor/acceptor)
cholesterol	none	6.1 ± 0.2	3.9 ± 0.2
cholesterol	diamide	6.1 ± 0.1	4.1 ± 0.4
phospholipid[b]	none	17.6 ± 1.0	11.3 ± 0.3
phospholipid[b]	diamide	9.5 ± 1.1	5.3 ± 0

[a] The values are the average ± SD of three to four experiments with separate donor and acceptor preparations. Acceptor SUV were prepared in STM buffer from egg PC (74.5 mol %), cholesterol (25.5 mol %), and a trace of [^{3}H]glycerol trioleate (0.5 μCi). The acceptor was present in 20-fold excess with respect to lipid concentration. The mycoplasma cell membranes and SUV contained the same cholesterol/phospholipid molar ratio. [b] Dimethyl sulfoxide was added (3% v/v, final concentration) to the incubation medium to enhance the rate of spontaneous ^{14}C-labeled phospholipid exchange. The membrane lipids of *M. gallisepticum* were labeled by growing the cells with 0.002 μCi of [^{14}C]palmitic acid/mL of medium; under these conditions, [^{14}C]-phosphatidylglycerol comprised about 95% of the total radiolabeled phospholipids.

remaining in the supernatant SUV. The half-times for the movement of radiolabeled cholesterol between SUV and mycoplasma cells are 1.3 and 2.2 h at 37 °C when α,γ-PDIT-mono-PE and -di-PE derivatives are present, respectively (Table I). The half-time for cholesterol exchange from SUV to mycoplasma cells is also increased significantly by incorporation of nitrosulfone-di-PE, but the increase is much larger in the SUV/SUV exchange system.

Diamide-Treated Mycoplasma Membranes. When *M. gallisepticum* cells (2 mg of cell protein) were treated with diamide (5 mM) for 1 h at 37 °C, many of the membrane proteins were cross-linked to such an extent that they did not penetrate into the SDS–polyacrylamide gels. Table II shows that the rate of [^{14}C]cholesterol exchange from *M. gallisepticum* intact cells or isolated membranes to SUV was not affected by diamide treatment. However, the same conditions of diamide treatment caused large enhancements in the initial rates of ^{14}C-labeled phospholipid exchange. The rate enhancements relative to untreated mycoplasma are 86% in the cell/SUV exchange system and 113% in the mycoplasma membrane/SUV exchange system.

Diamide-Treated Erythrocyte Ghosts. Ghosts were treated with diamide to give extensive cross-linking of spectrin as well as cross-linking of many of the other major membrane proteins. These ghosts exhibited increases in the exchange rates of both [^{14}C]cholesterol (Figure 4) and [^{14}C]PC (Figure 5). The half-times for labeled cholesterol exchange from untreated or diamide-treated ghosts to SUV are 2.9 ± 0.2 and 2.4 ± 0.1 h, respectively. For [^{14}C]PC exchange (in the presence of dimethyl sulfoxide, which facilitated the intermembrane movement), the $t_{1/2}$ values are 9.5 ± 0.4 and 6.1 ± 0.2 h in the untreated and diamide-treated ghosts, respectively. Thus the exchange rate of [^{14}C]PC is enhanced to a greater extent than that of [^{14}C]cholesterol by diamide treatment of erythrocyte ghosts.

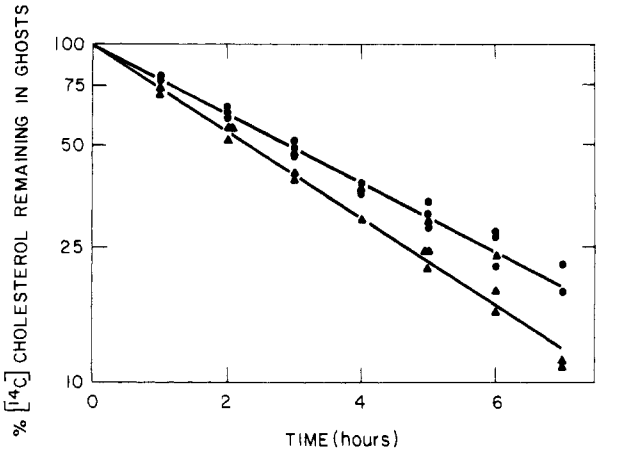

FIGURE 4: Effect of diamide on kinetics of [^{14}C]cholesterol exchange from erythrocyte ghosts to egg PC/cholesterol SUV. Erythrocyte ghosts (2 mg of protein/mL) were treated with diamide (5 mM final concentration) in PBS buffer at 37 °C for 1 h and then collected and washed as described under Materials and Methods. Untreated ghosts (●); diamide-treated ghosts (▲). Erythrocyte ghosts and SUV (cholesterol/phospholipid molar ratio, 0.9–1.0) were incubated in PBS buffer containing 1% (w/v) albumin.

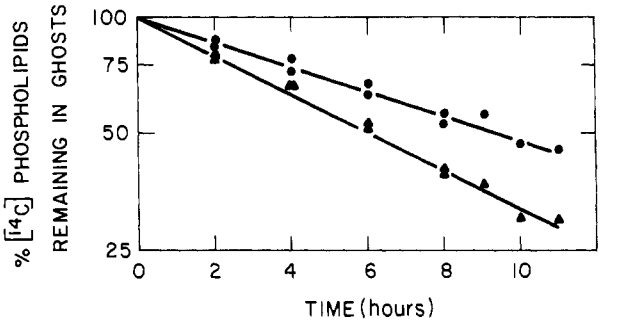

FIGURE 5: Effect of diamide on kinetics of spontaneous [^{14}C]-phospholipid exchange from erythrocyte ghosts to egg PC/cholesterol SUV. The conditions and symbols are the same as in Figure 4, except that dimethyl sulfoxide was also included in the incubation medium (3% v/v).

Discussion

In this paper we show that the presence of cross-linked PE derivatives inhibits the rate of cholesterol exchange between membranes, whereas the formation of an extensive number of disulfide bonds in membrane proteins enhances the rate of phospholipid exchange from the two plasma membranes (mycoplasma and erythrocyte) we investigated. Our finding that the [^{14}C]PC exchange rate is enhanced in diamide-treated erythrocyte ghosts agrees with the enhanced rates of transbilayer movement of lipids reported by other investigators (Haest et al., 1978; Franck et al., 1982; Mohandas et al., 1982). However, the rate of [^{14}C]cholesterol exchange was not altered when *M. gallisepticum* cells or membranes were treated with diamide (Table II). Since these conditions of diamide treatment gave a doubling of the phospholipid exchange rate, it is apparent that cholesterol and phosphatidylglycerol are not cotranslocated. These results suggest that the membrane structural defects resulting from diamide-induced oxidative cross-linking of sulfhydryl groups (Deuticke et al., 1983) are localized and that lipids may undergo transbilayer movement at relatively discrete reorientation sites, as proposed in erythrocyte membranes by Bergmann et al. (1984). The proteins in the vicinity of cholesterol molecules undergoing exchange from *M. gallisepticum* appear to be less reactive than those adjacent to phospholipids. The results on diamide treatment of erythrocyte ghosts (Figures 4 and 5) also show a greater enhancement of [^{14}C]PC than of [^{14}C]cholesterol exchange rate (56 and 25%, respectively), which also indicates that disulfide bond formation does not lead to random defects throughout the membrane.

The finding that [^{14}C]PC undergoes spontaneous exchange from ghosts to SUV more slowly than [^{14}C]cholesterol (in the presence of dimethyl sulfoxide, Table II) agrees with observations obtained with SUV (Bloj & Zilversmit, 1977; Jonas

& Maine, 1979; McLean & Phillips, 1981) and mycoplasmas (Clejan & Bittman, 1984) as the donor species. Exchange of radiolabeled cholesterol from erythrocytes appears to take place via the aqueous phase (Lange et al., 1983). The rate of lipid exchange depends on the aqueous solubility of the lipid (McLean & Phillips, 1981; Clejan & Bittman, 1984; Bruckdorfer & Sherry, 1984, and references cited therein). The increases in $t_{1/2}$ values of [^{14}C]cholesterol on incorporation of cross-linked PE derivatives into donor SUV probably result from increased lipid order; model and biological membranes that have been subjected to lipid peroxidation show decreased lipid mobility (Pauls & Thompson, 1980; Vladimirov et al., 1980; Rice-Evans & Hochstein, 1981; Fukuzawa et al., 1981; Eichenberger et al., 1982). Interactions between cholesterol and its nearest neighbors are apparently increased when dimeric PE derivatives are present. Thus, the dissolution of cholesterol from the surface of donor SUV becomes more difficult.

It is of interest to consider the possible significance of the observation that a variety of cross-linked PE derivatives lower the rate of cholesterol exchange. In vitro studies have shown that treatment of normal erythrocytes with malondialdehyde results in inhibition of [^{14}C]cholesterol uptake from radiolabeled plasma (Jain & Shohet, 1982). Irreversibly sickled cells also have a decreased rate of cholesterol exchange with plasma cholesterol (Jain & Shohet, 1982). Since the amount of malondialdehyde in sickled cells is higher than that in normal cells, the adducts formed between amino groups of proteins and/or phospholipids with malondialdehyde (Jain & Hochstein, 1980) may be responsible for the decreased cholesterol exchangeability. Our results suggest that cross-linking between two phospholipid molecules may have physiological significance, since we found that these molecules decrease the rate of cholesterol translocation. In fact, Jain & Shohet (1984) recently detected an adduct in erythrocytes in which the amino groups of phospholipids are cross-linked via Schiff base linkages. The experimental approach of introducing synthetic cross-linked lipids into membranes used in this report avoids numerous other processes that can accompany lipid peroxidation, such as decreases in the number of double bonds in fatty acyl chains, formation of conjugated and trans double bonds, and reactions between neighboring fatty acyl chains. However, since our results are not directly applicable to erythrocytes, we cannot exclude the possibility that cross-linking of phospholipids with proteins also contributes to inhibition of cholesterol exchange in sickle cells.

Registry No. Nitrosulfone-mono-PE, 94751-63-0; nitrosulfone-di-PE, 94751-64-1; DL-α,β-DPPE, 5681-36-7; DL-α,γ-DPPE, 94780-96-8; FNPS, 312-30-1; α,β-PDIT-mono-PE, 94751-66-3; α,γ-PDIT-mono-PE, 94751-69-6; PDIT, 4044-65-9; α,β-PDIT-di-PE, 94751-67-4; α,γ-PDIT-di-PE, 94751-65-2; 1,4-xylylene-di-PE, 94751-68-5; terephthalaldehyde, 623-27-8; cholesterol, 57-88-5; diamide, 10465-78-8.

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