Calcium/Phosphate-Induced Immobilization of Fluorescent Phosphatidylserine in Synthetic Bilayer Membranes: Inhibition of Lipid Transfer between Vesicles†

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Received October 29, 1985; Revised Manuscript Received December 18, 1985

ABSTRACT: Resonance energy transfer between 4-nitro-2,1,3-benzoxadiazole (NBD) acyl chain labeled phospholipid analogues and (lissamine) rhodamine B labeled phosphatidylethanolamine was used to monitor the rate of NBD-labeled lipid transfer between a variety of small unilamellar donor vesicles and dioleoylphosphatidylcholine (DOPC) acceptor vesicles. In the presence of appropriate concentrations of Ca^{2+} and phosphate, the transfer rate of NBD-phosphatidylserine (NBD-PS) from vesicles composed of lipid extracts from human red blood cells was reduced by ~10-fold, while the transfer rates of NBD-phosphatidylcholine, -ethanolamine, -glycerol, -*N*-succinylethanolamine, and -phosphatidic acid were essentially unaffected. A systematic evaluation of the lipid composition needed to facilitate the Ca^{2+}/phosphate-induced inhibition of NBD-PS transfer revealed that the process was dependent upon the inclusion of both cholesterol and phosphatidylethanolamine (PE) in the donor vesicle population. Inhibition of NBD-PS transfer required the sequential addition of phosphate and Ca^{2+} to the vesicles, indicating that the combined interaction of Ca^{2+} and phosphate at the membrane surface was a prerequisite for inhibition to occur. Parallel experiments designed to determine the possible mechanism of this phenomenon showed that inhibition of NBD-PS transfer was not due to Ca^{2+}-mediated phase separations or vesicle–vesicle fusion. However, the addition of Ca^{2+} and phosphate to vesicles composed of total red blood cell lipids or cholesterol/PE did result in their aggregation. On the other hand, aggregation per se did not seem to be responsible for the inhibition of transfer since NBD-PS-containing vesicles composed of DOPC or DOPC/DOPE also aggregated, although NBD-PS transfer was unaffected. Our data show that PS can be immobilized by Ca^{2+} and phosphate in model bilayer membranes containing cholesterol and PE. These results suggest that Ca^{2+} and phosphate might induce the formation of intramembrane complexes with PS. The potential implication of such a mechanism for the maintenance of PS asymmetry in biological membranes is discussed.

Many biological and biochemical studies have demonstrated that certain phospholipid molecules can transfer or exchange between lipid vesicles and between vesicles and cells. Although this phenomenon has been shown to occur with unmodified lipids (Papahadjopoulos et al., 1976; Martin & MacDonald, 1976; De Cuyper et al., 1980, 1983), recent studies using fluorescent acyl chain labeled phospholipid analogues have proven to be invaluable in the study of spontaneous lipid monomer diffusion (Roseman & Thompson, 1980; Nichols & Pagano, 1981, 1982, 1983) and in the study of lipid translocation and metabolism in in vitro cultivated cells (Pagano & Sleight, 1985). In a number of these systems it has been shown that a substantial fraction of the NBD[1]-labeled lipids transferred into cell membranes can be removed via a process known as "back exchange" (Struck & Pagano, 1980), which, in several instances, has been shown to correspond to the amount of lipid residing in the external leaflet of the cells (Struck & Pagano, 1980; Pagano & Sleight, 1985).

Using similar techniques of lipid transfer, we have recently reported that once NBD-PS (acyl chain labeled) is transferred to red blood cells (RBC), it can no longer be removed from the cells, in contrast to other similarly labeled lipids such as NBD-PC and NBD-PG (Tanaka & Schroit, 1983; Schroit et al., 1985). Although several reports have recently suggested that a spin-labeled PS analogue in RBC is susceptible to ATP-dependent bilayer translocation (Seigneuret & Devaux, 1984; Seigneuret et al., 1984), our previous results have shown that a substantial fraction of NBD-PS in RBC was localized in the outer leaflet of the cells as determined by its accessibility to derivatization by trinitrobenzenesulfonate (Schroit et al., 1985).

Taking into consideration the recent observations on the role of PS in Ca^{2+}-induced bilayer dehydration (Portis et al., 1979; Düzgüneş et al., 1984; Hoekstra, 1982a,b), phase separation (Hoekstra, 1982a; Tilcock et al., 1984), and its avid association with integral membrane components (Sato & Ohnishi, 1983; Ong, 1984; Mendelsohn et al., 1984), it is not unlikely that normally transferable NBD-PS might be physically restricted by one or more of these processes. Indeed, a recent report by

† This work was supported in part by Developmental Fund Grant 175416 from The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston and by National Institutes of Health Grant CA-40149.

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[1] Abbreviations: ^{125}I-PE, *N*-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)-propionyl]dipalmitoylphosphatidylethanolamine; Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; egg PE, transphosphatidylated egg phosphatidylcholine; NBD, 4-nitro-2,1,3-benzoxadiazole; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; NBD-PC, -PS, -PG, -PE, and -COOH, 1-acyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine, -serine, -glycerol, -ethanolamine, and -*N*-succinylethanolamine; NBD-PA and -DG, 1-acyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidic acid and -diglyceride; RBC, red blood cells; TLE, human RBC total lipid extract; NaCl–HEPES, 150 mM NaCl–10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-2141$01.50/0

Raval and Allen (1984) has indicated that, although there is a partial loss in the transmembrane distribution of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in spectrin-free vesicles derived from RBC, the asymmetric distribution of PS remained essentially unchanged.

In an effort to determine if NBD-PS can be "immobilized" in a bilayer membrane, we have employed previously described techniques of resonance energy transfer (Fung & Stryer, 1978; Struck et al., 1981) to measure the rate of phospholipid transfer (Nichols & Pagano, 1982, 1983) and the size of the exchangeable pool (Pagano et al., 1981) in model membrane systems. Our results indicate that NBD-PS can undergo an apparent calcium/phosphate-mediated, PE/cholesterol-dependent immobilization in synthetic bilayer membranes, which is not associated with bilayer translocation of the lipid analogue.

Materials and Methods

Methods and Routine Procedures. DOPC, DOPE, N-NBD-PE, Rho-PE, and NBD-PC were purchased from Avanti Polar Lipids (Birmingham, AL). ^{125}I-PE was synthesized as described previously (Schroit, 1982). Phospholipase D (cabbage) was obtained from Boehringer-Mannheim and phospholipase C (*Clostridium perfringens*) from Calbiochem. All lipids were stored at −70 °C and were monitored by thin-layer chromatography using activated silica gel 60 thin-layer plates (Merck). Lipid concentrations were determined according to Ames and Dubin (1960). RBC total lipid extracts (TLE) were prepared from washed human RBC ghosts (Steck & Kant, 1974) by using the 2-propanol extraction procedure of Rose and Oaklander (1965).

Fluorescent Lipids. NBD-labeled lipids were prepared by enzymatic hydrolysis or base exchange from the corresponding NBD-PC. Briefly, NBD-PS, NBD-PG, and NBD-PE were prepared by phospholipase D catalyzed base exchange in the presence of L-serine, glycerol, and ethanolamine, respectively. NBD-PA was isolated as a byproduct from the NBD-PC/ethanolamine reaction mixture. NBD-DG was prepared from NBD-PC with phospholipase C (Kates, 1972). *N*-Succinyl-NBD-PE (NBD-COOH) was prepared from NBD-PE by condensation with succinic anhydride as described previously (Nayar & Schroit, 1985). All of the derivatives were purified by thin-layer chromatography in appropriate solvent systems. Analysis of the purified phospholipid products revealed single fluorescent and phosphate-positive spots. The amino-containing lipids were positively identified on the basis of their reactivity with picrylsulfonic acid by assessing their altered mobility on thin-layer chromatography plates.

Vesicle Preparation. Vesicles were prepared from appropriate lipid mixtures following removal of the solvents by evaporation under nitrogen and high vacuum overnight. Small unilamellar acceptor vesicles were prepared by the ethanol-injection technique (Kremer et al., 1977) by dissolving 10 mg of DOPC in 3.34 mL of ethanol, followed by rapid injection into 46 mL of HEPES-buffered saline (pH 7.4) and overnight dialysis against 5 L of HEPES–saline at 4 °C (final lipid concentration = 217 μg/mL). Small unilamellar donor vesicles were prepared by ethanol injection or by ultrasonication of appropriate carrier lipid mixtures containing 1% Rho-PE and 1% of the NBD lipid analogue under study.

Determination of Vesicle Aggregation. Vesicle aggregation was determined by assessing the ability of the various preparations to precipitate at 10000*g*. For these experiments vesicles were formed as described above except for the addition of trace amounts of ^{125}I-PE. Briefly, incubations were performed in an Eppendorf microfuge tube (1.5-mL capacity), and a 25-μL aliquot of the suspension was removed and counted. Following centrifugation for 5 min, a 25-μL aliquot of the supernatant was removed and counted. The fraction of vesicles precipitated was estimated by using the equation (total cpm − supernatant cpm)/total cpm.

Fluorescence Measurements. Steady-state fluorescence was quantified at 530 nm (λ_{ex} 450 nm) with a dual-beam Farrand MKII spectrophotofluorometer at 25 °C in the differential mode using 2.5-nm excitation and emission slits. Data acquisition was controlled by an Apple IIe computer that sampled and digitized data from the fluorometer at 50-ms intervals. The initial rates of lipid transfer were obtained from the initial linear slope of the fitted curves from computer-generated curve-fitting routines. The amount of NBD-lipid relative fluorescence was determined after the addition of 2% Triton X-100 (w/w final concentration) and compared to a standard curve generated from known amounts of standard NBD-lipid in the same amount of total nonfluorescent lipid at the same temperature. The fluorescent yield of the various NBD-lipids in the Triton X-100 lysates (as compared to the fluorescent yield of the lipids in the vesicles) was determined independently for each analogue by measuring the fluorescence of DOPC vesicles containing 0.1% NBD-lipid before and after the addition of detergent and was about half that of vesicles containing nonquenching concentrations of the analogue (Tanaka & Schroit, 1983, and Figure 4).

Resonance Energy Transfer Assay. The resonance energy transfer assay for measuring the transfer of NBD-labeled lipids between two vesicle populations was carried out essentially as described by Nichols and Pagano (1982, 1983). Briefly, the energy-transfer efficiency (Struck et al., 1981) of the initial donor vesicle population was determined by measuring the fluorescence of a 10-μL aliquot of the vesicles in 1.3 mL of HEPES–saline before and after the addition of detergent (with appropriate solutions in the reference cuvette). Typically, values of 90–95% were obtained. Transfer rates and the amount of totally exchangeable lipid were routinely determined by rapidly mixing 1 mL of unlabeled acceptor vesicles with 10 μL of donor vesicles (donor/acceptor lipid ratio of 1/100) in 300 μL of HEPES–saline containing appropriate concentrations of sodium phosphate, $CaCl_2$, or $MgCl_2$; the same concentration of acceptors alone was used in the reference cuvette. Initial rates of transfer were determined from the initial linear slope of fluorescence enhancement (relief of NBD-lipid quenching by Rho-PE; Nichols & Pagano, 1982) whereas the total exchangeable pool was determined after the fluorescence reached equilibrium (Pagano et al., 1981).

Results

Inhibition of NBD-PS Transfer. The resonance energy transfer assay for measuring the transfer of NBD-labeled lipids between different vesicle populations is based upon previously described procedures (Nichols & Pagano, 1982, 1983). Briefly, with a population of donor vesicles containing approximately 1 mol % each of Rho-PE and NBD-lipid, resonance energy transfer between the NBD-lipid and Rho-PE results in significant NBD quenching. Upon the addition of unlabeled acceptor vesicles, the NBD-lipids can spontaneously transfer to the acceptor membranes while the Rho-PE remains associated with the donor population (Struck et al., 1981). As the NBD-lipid transfers to the acceptor vesicles, it is no longer quenched and an increase in NBD emission is observed. The transfer rate can then be determined from the initial linear slope of fluorescent development (see Materials and Methods).

Figure 1 shows the development of NBD fluorescence (an indication of transfer rate) from NBD-PC and NBD-PS in

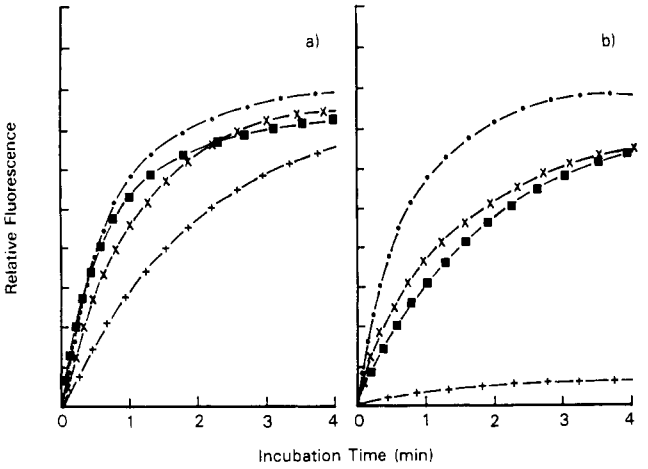

FIGURE 1: Transfer of NBD-PC and NBD-PS between vesicles. Donor vesicles (10 μL; 2.2 μg of lipid) composed of (a) NBD-PC/Rho-PE/DOPC (1/1/98) and NBD-PS/Rho-PE/DOPC (1/1/98) or (b) NBD-PC/Rho-PC/TLE (1/1/98) and NBD-PS/Rho-PE/TLE were added to 300 μL of NaCl–HEPES containing 1.0 μmol of sodium phosphate in a 1-cm cuvette. $CaCl_2$ was then added, and after a 3-min incubation period, 1.0 mL of acceptor vesicles (220 μg of lipid) was rapidly mixed with the donor suspension and the development of fluorescence was recorded. The final phosphate and calcium concentrations were 3.3 and 5 mM, respectively. NBD-PC without calcium (●); NBD-PC with calcium (■); NBD-PS without calcium (×); NBD-PS with calcium (+).

donor vesicles containing Rho-PE/DOPC (Figure 1a) and Rho-PE/TLE (Figure 1b) in phosphate-containing buffer (3.3 mM) upon the addition of a 100-fold excess of unlabeled acceptor vesicles in the presence and absence of 5 mM Ca^{2+}. It can be seen that Ca^{2+} had no effect on the transfer of NBD-PC from DOPC donors, while the transfer of NBD-PS decreased by ~2-fold under the same conditions. On the other hand, the addition of Ca^{2+} to donor vesicles composed of TLE resulted in a 2-fold decrease in the transfer rate of NBD-PC and more than a 10-fold decrease in the transfer rate of NBD-PS. These initial observations suggested that calcium, in combination with phosphate, can inhibit the intervesicular transfer of normally transferable NBD-PS when incorporated into a membrane of appropriate lipid composition.

In order to determine the concentration of calcium and phosphate required to inhibit NBD-PS transfer, NBD-PS/Rho-PE/TLE donor vesicles were mixed with acceptor vesicles

Table I: Effect of Sequence of Ca^{2+} and Phosphate Addition on NBD-PS Transfer[a]

buffer[b]	addition[b]	transfer rate (ng/min)
vesicles		3.85
vesicles + phosphate		4.06
	Ca^{2+}	0.51
vesicles + calcium		3.21
	phosphate	0.58
phosphate + calcium	vesicles	2.78

[a] Vesicles were preincubated with the indicated compounds (in sequence) for 2 min. Acceptor vesicles were then added (1 mL; 200 μg) and the initial transfer rate was determined. [b] The phosphate and Ca^{2+} concentrations used were 3 and 5 mM, respectively.

in the presence of different Ca^{2+} and phosphate concentrations (Figure 2). In the absence of phosphate, increasing Ca^{2+} concentrations resulted in only small reductions in transfer rates, with 10 mM Ca^{2+} reducing the rate by only ~2-fold (Figure 2a). Significant decreases did occur, however, in the presence of 0.33 mM phosphate, where 2.5 and 5.0 mM Ca^{2+} reduced the transfer rate by a factor of 3 and 10 mM Ca^{2+} reduced the transfer rate by 10 times (Figure 2b). In the presence of 3.3 mM phosphate, inhibition of transfer was dramatic, with 5 mM and 10 mM Ca^{2+} resulting in 10-fold and 30-fold decreases in the transfer rate, respectively (Figure 2c).

To further evaluate the Ca^{2+}/phosphate requirements for inhibition of NBD-PS transfer, NBD-PS/TLE vesicles were either premixed with phosphate (as above), with Ca^{2+} followed by phosphate, or added to premixed Ca^{2+}/phosphate. As shown in Table I, inhibition of NBD-PS transfer required both phosphate and Ca^{2+} regardless of which component was added to the vesicles first. On the other hand, mixing Ca^{2+} with phosphate prior to the addition of vesicles did not result in any significant decrease in the rate of NBD-PS transfer.

Since specific pH-dependent Ca^{2+}/phosphate complexes are known to form with PS (Cotmore et al., 1971; Fraley et al., 1980), this parameter was investigated for its effect on the rate of NBD-PS transfer. The results shown in Figure 3a indicate no significant alterations in NBD-PS transfer at pH 8.0, 7.4, or 6.8, which suggests that pH per se has little or no effect on spontaneous NBD-PS transfer. However, transfer could not be inhibited by Ca^{2+} (5 mM) at pH 6.8 in contrast to the marked inhibition that occurred at both pH 7.4 and 8.0 (Figure

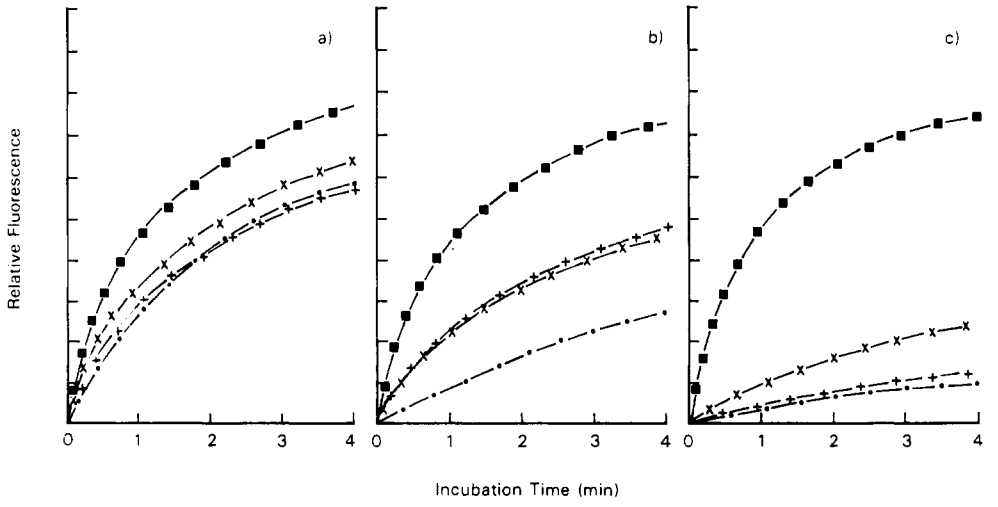

FIGURE 2: Dependence of calcium and phosphate concentrations on inhibition of NBD-PS transfer. NBD-PS/Rho-PE/TLE donor vesicles (2.2 μg) were added to 300 μL of NaCl–HEPES containing (a) 0, (b) 0.1, or (c) 1.0 μmol of sodium phosphate. Concentrated solutions of $CaCl_2$ were then added, and after a 3-min incubation period, 1 mL of DOPC acceptor vesicles (220 μg) was rapidly mixed with the donor suspension. The final calcium and phosphate concentrations were 0 (■), 2.5 (×), 5.0 (+), and 10 mM (●) and (a) 0, (b) 0.33, and (c) 3.3 mM, respectively.

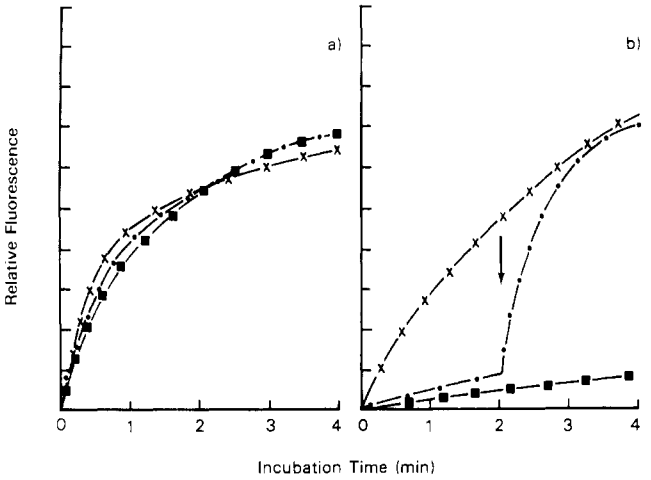

FIGURE 3: pH dependence of calcium/phosphate inhibition of NBD-PS transfer. Acceptor DOPC vesicles were mixed with NBD-PS/Rho-PE/TLE (1/1/98) donor vesicles previously equilibrated against NaCl–HEPES and titrated with HCl or NaOH to the indicated pH. (a) Transfer kinetics in NaCl–HEPES with phosphate; (b) transfer kinetics in NaCl–HEPES containing phosphate and calcium (see legend to Figure 1). pH: 6.8 (×); 7.4 (●); 8.0 (■). In (b), a concentrated solution of EDTA was added after 2 min (arrow).

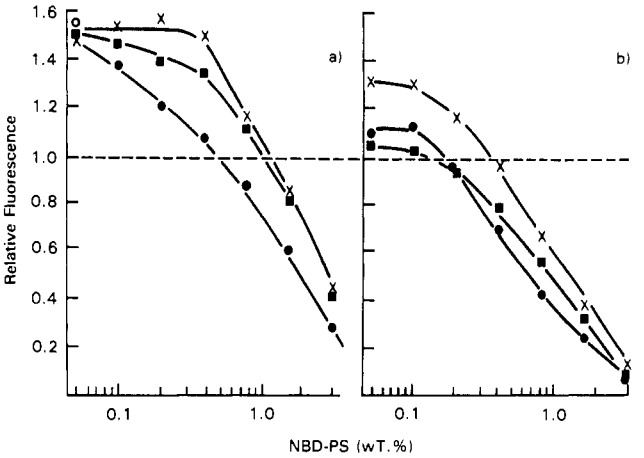

FIGURE 4: Calcium/phosphate induced self-quenching of NBD-PS. Donor vesicles containing increasing amounts of NBD-PS in (a) DOPC or (b) TLE (carrier lipids kept constant) were incubated in NaCl–HEPES (■), NaCl–HEPES containing 1 mM EDTA (×), or NaCl–HEPES containing sodium phosphate (3.3 mM) and $CaCl_2$ (5 mM) (●). The fluorescence was measured before and after the addition of Triton X-100 (2% final concentration). The values obtained following the addition of Triton X-100 were corrected for sample dilution and normalized to 1 (dashed horizontal line).

3b). As might be expected, the addition of 10 mM EDTA (arrow) resulted in an apparent immediate resumption in NBD-PS transfer to its spontaneous rate.

Although the results presented above suggest that Ca^{2+}/phosphate can indeed inhibit the transfer of NBD-PS, it is possible that these observations might also have been due to alternative mechanisms such as self-quenching of NBD-PS and/or fusion of the donor vesicles, which conceivably could induce lipid rearrangements and result in different "local" NBD-PS densities. To rule out these possibilities, we examined (1) the potential for Ca^{2+}/phosphate to induce self-quenching of NBD-PS in both DOPC and TLE vesicles in the absence of Rho-PE (since Rho-PE quenches NBD fluorescence) and (2) the fusogenic potential of donor TLE vesicles containing the two nonexchangeable probes, N-NBD-PE and Rho-PE, in the presence of excess unlabeled TLE vesicles.

(1) Donor vesicles containing different concentrations of NBD-PS in DOPC or TLE were incubated in buffer alone, buffer containing 1 mM EDTA, or buffer containing phosphate (3.3 mM) and Ca^{2+} (5 mM), and the fluorescence was monitored. The results presented in Figure 4 show that typical density-dependent quenching curves are obtained (normalized to Triton values of 1), which indicate that Ca^{2+}/phosphate induces only nominal and similar self-quenching throughout the range of 0.05–3.2 mol % NBD-PS in both the DOPC and TLE vesicles. For example, at 1 mol % NBD-PS, the ratio of fluorescence in EDTA vs. Ca^{2+}/phosphate is 1.4 and 1.5 for DOPC and TLE vesicles, respectively. These results suggest that the inability of NBD-PS to transfer from the TLE vesicles in the presence of Ca^{2+}/phosphate was not due to a specific TLE-dependent self-association of NBD-PS in the membrane.

(2) Ca^{2+}/phosphate-induced vesicle–vesicle fusion (Fraley et al., 1980) was monitored by the resonance energy transfer technique (Struck et al., 1981), which has been shown to be an ideal method for determining membrane intermixing. However, since homotypic fusion would be undetectable in our system (the TLE vesicles contain identical amounts of fluorescent lipid analogues), these experiments were carried out by mixing aliquots of TLE vesicles containing both N-NBD-PE (the energy donor) and Rho-PE (the energy acceptor) with increasing amounts of unlabeled TLE vesicles.[2] The extent

Table II: Fusion of N-NBD-PE/Rho-PE/TLE Vesicles with TLE Vesicles[a]

unlabeled/labeled vesicle ratio	observed		expected[d]	
	efficiency[b]	Rho-PE density[c]	efficiency	Rho-PE density
0	86.3	0.0171	86.3	0.0171
1	82.9	0.0142	71.5	0.0086
2	83.7	0.0149	57.0	0.0057
4	74.1	0.0094	38.9	0.0034
5	74.5	0.0095	34.1	0.0029
7.5	77.1	0.0105	24.7	0.0020
10	80.1	0.0121	20.2	0.0016
20	76.7	0.0104	10.6	0.0008

[a] N-NBD-PE/Rho-PE/TLE vesicles (2 μg) were mixed with increasing amounts of unlabeled vesicles (0–40 μg) in 300 μL of NaCl–HEPES containing 1.0 μmol of sodium phosphate in a 1-cm cuvette. $CaCl_2$ was then added (1.5 μmol), and after a 5-min incubation period, 1 mL of buffer was added and the fluorescence intensity at 530 nm was recorded (λ_{ex} 450 nm). [b] Energy-transfer efficiency was calculated from the relationship $E = 1 - F/F_0$, where F and F_0 are the relative fluorescence intensities in the presence and absence of detergent (Struck et al., 1981). [c] E was converted to the observed Rho-PE densities by using an appropriately generated standard curve [see text and Struck et al. (1981)]. [d] The expected efficiencies and Rho-PE densities were estimated by assuming that complete lipid randomization occurs during fusion.

of fusion was estimated by measuring the increase in fluorescent yield of the N-NBD-PE, which occurs as a result of reduced energy-transfer efficiency caused by an effective decrease in the energy acceptor's density upon fusion with unlabeled vesicles (Struck et al., 1981). The addition of Ca^{2+}/phosphate to mixtures of fluorescent and unlabeled vesicles resulted in an immediate decrease in the measured energy-transfer efficiency. Using an appropriately generated standard curve for fluorescent yield based on the density of the energy acceptor [see Struck et al. (1981) for details], we estimate that the surface density of the Rho-PE 15 min after the addition of Ca^{2+}/phosphate decreased from 0.017 (the

[2] It should be noted that vesicles containing NBD-PS could not be used for this purpose since the rapid transfer properties of the lipid (before the addition of Ca^{2+}/phosphate) would have obscured any possible fusion events.

Table III: Specificity of Calcium/Phosphate Inhibition of NBD-PS Transfer[a]

donor vesicles[c]	initial transfer rate[b] buffer	+Ca^{2+}	+Mg^{2+}	initial rate decrease (−Ca^{2+}/+Ca^{2+})	% lipid transferred[d]
NBD-PC/DOPC	5.7	5.4	5.6	1.1	0.65
NBD-PS/DOPC	3.7	2.0	4.6	1.9	0.56
NBD-DG/DOPC	0.4	0.4		1.0	1.01
NBD-PC/TLE	5.8	5.5	5.7	1.1	0.62
NBD-PE/TLE	2.8	2.2	2.3	1.3	0.55
NBD-PS/TLE	2.9	0.2	2.0	14.5	0.58
NBD-PG/TLE	6.3	5.8	5.2	1.1	
NBD-PA/TLE	6.0	5.5	4.6	1.3	
NBD-COOH/TLE	5.2	4.8	4.4	1.2	

[a] DOPC acceptor vesicles (1 mL; 202 μg of lipid) were rapidly mixed with donor vesicles (2 μg) in NaCl–HEPES containing 3.3 mM sodium phosphate and 5 mM $CaCl_2$ (see Figure 1 legend), and the initial rate of transfer was recorded. [b] Initial rates are expressed as the actual amount of lipid transferred/min based on the fluorescence of standard vesicle suspensions in Triton X-100 (see Materials and Methods). [c] All donor vesicles contained NBD-lipid/Rho-PE/TLE at a 1/1/98 ratio. [d] The exchangeable lipid pool in the vesicles was estimated by the ratio of the initial energy-transfer efficiency of the donor vesicles in buffer alone (E_1) and the energy-transfer efficiency of the donor vesicles after equilibration (>8-h incubation) with a 100-fold excess of acceptor vesicles (E_2), where the fraction of lipid transferred equals $1 - (E_2/E_1)$.

initial density) to a minimum of 0.0093 (Table II). In addition, the results presented in Table II show that increasing amounts of unlabeled acceptors did not result in a corresponding decrease in probe density more than that expected for the fusion of 1 fluorescent vesicle with 0.8 unlabeled vesicle (obtained from the expected densities assuming complete lipid intermixing). This can be seen from the observed energy-transfer efficiencies and the corresponding observed Rho-PE densities required to maintain these efficiencies (obtained from the standard curve), which are incompatible with the expected energy-transfer efficiencies and the corresponding expected densities if complete lipid intermixing had occurred. From these results we conclude that homotypic fusion is not responsible for the observed reduction in the rate of NBD-PS transfer in the presence of Ca^{2+}/phosphate.

Specificity of Calcium/Phosphate-Mediated Inhibition of NBD-PS Transfer. In order to test the specificity of the observed inhibition in NBD-PS transfer, similar experiments were carried out with a variety of other neutral and negatively charged acidic phospholipids. The results of these experiments expressed as the actual initial rate of transfer (nanograms of lipid per minute) are presented in Table III. The results indicate that Ca^{2+}/phosphate-mediated inhibition of lipid transfer was indeed specific for NBD-PS, resulting in a 14-fold decrease in rate, whereas the other acidic phospholipids tested, NBD-PG, NBD-PA, and NBD-COOH (succinylated NBD-PE), were essentially unaffected. In addition, we assessed the ability of the various lipids to undergo transbilayer movement by adding increasing amounts of acceptor vesicles and determining the size of the exchangeable lipid pool [see Struck et al. (1981) for details]. The results also presented in Table III show that ~60% of NBD-PC, NBD-PE, and NBD-PS was localized in the outer leaflet of the donor vesicles irrespective of carrier lipid composition (DOPC or TLE), which suggested that NBD-PS did not selectively partition to one of the bilayer leaflets. As a control and in agreement with previously published data (Ganong & Bell, 1984; Pagano & Longmuir, 1985), diglyceride underwent complete transbilayer movement.

Table IV: Transfer of NBD-PS from Donor Vesicles of Defined Lipid Compositions[a]

donor vesicles[b]		transfer rate[c]	−Ca^{2+}/+Ca^{2+}
DOPC		5.29	
	+Ca^{2+}	4.59	1.2
TLE		4.15	
	+Ca^{2+}	0.25	16.6
DOPC/Chol (50/50)		2.38	
	+Ca^{2+}	1.56	1.5
DOPC/DOPE (80/20)		3.88	
	+Ca^{2+}	2.23	1.7
DOPC/DOPE (50/50)		1.98	
	+Ca^{2+}	0.51	3.9
DOPC/Chol/DOPE (25/50/25)		0.83	
	+Ca^{2+}	0.10	8.3
DOPC/Chol/DOPE (40/50/10)		1.80	
	+Ca^{2+}	0.18	10.0
DOPC/Chol/DPPE (40/50/10)		2.25	
	+Ca^{2+}	0.27	8.4
DOPC/Chol/egg PE (40/50/10)		1.50	
	+Ca^{2+}	0.14	10.7

[a] DOPC acceptor vesicles (1 mL; 202 μg of lipid) were rapidly mixed with donor vesicles (2 μg) in NaCl–HEPES containing 3.3 mM sodium phosphate and 5 mM $CaCl_2$ (see Figure 1 legend), and the initial rate of transfer was recorded. [b] All donor vesicles contained 1% NBD-PS and Rho-PE. [c] Initial rates are expressed as the actual amount of lipid transferred/min based on the fluorescence of standard vesicle suspensions in Triton X-100 (see Materials and Methods).

Interestingly, the addition of 5 mM Mg^{2+}, which has been shown to have dramatic effects on phosphatidylserine in other systems, was incapable of inhibiting the transfer of NBD-PS.

Lipid Requirements for Inhibition of NBD-PS Transfer. Since DOPC alone was insufficient in promoting significant Ca^{2+}/phosphate-mediated inhibition of NBD-PS transfer, it was likely that other auxiliary lipids present in the RBC lipid extracts were required in order for this process to occur.

To try to determine which lipid components in the human RBC total lipid extract were responsible for mediating the Ca^{2+}/phosphate inhibition of NBD-PS transfer, donor vesicles of defined lipid composition were made and the transfer kinetics of NBD-PS was determined. The results shown in Table IV indicate that cholesterol (DOPC/Chol, 50/50) or DOPE alone (DOPC/DOPE, 50/50) was ineffective in inhibiting the transfer of NBD-PS. On the other hand, donor vesicles consisting of DOPC/Chol/DOPE at either a 25/50/25 or 40/50/10 mole ratio resulted in the efficient inhibition of NBD-PS transfer with rates approaching that of the human RBC total lipid extract. Interestingly, the requirement for the inclusion of PE was independent of acyl-chain composition since DPPE and egg PE induced inhibition of NBD-PS transfer as well as did DOPE (Table IV). These results suggest, therefore, that inhibition of NBD-PS transfer is dependent upon the presence of PE and cholesterol, a finding remarkably similar to the auxiliary lipid requirements for subthreshold (in comparison with pure PS vesicles) concentrations of Ca^{2+} to fuse mixed PS/Chol (Uster & Deamer, 1981; Braun et al., 1985) or PS/PE vesicles (Uster & Deamer, 1981).

Ca^{2+}/Phosphate-Induced Vesicle Aggregation. Since it has been reported that Ca^{2+}- and/or Ca^{2+}/phosphate-induced vesicle fusion is always preceded by vesicle aggregation (Portis et al., 1979; Braun et al., 1985; Nir et al., 1980; Wilschut et al., 1981), we investigated whether aggregation might be associated with the observed phenomenon of Ca^{2+}/phosphate-induced inhibition of NBD-PS transfer. The results presented in Table V indicate that, indeed, those conditions that inhibited the transfer of NBD-PS also resulted in vesicle aggregation. However, it appears that vesicle aggregation per

Table V: Calcium/Phosphate Precipitation of Vesicles[a]

carrier lipid[b] composition	buffer	% vesicles precipitated				
			NBD-lipid analogue[c]			
		none	PC	PS	PE	COPE
DOPC	control	6	4	0	5	0
	$+Ca^{2+}$	4				
	$+Ca^{2+}/P$	6	3	16	8	3
TLE	control	12	7	6	7	7
	$+Ca^{2+}$	16	10	7		
	$+Ca^{2+}/P$	90	85	88	84	85
	$+Mg^{2+}$			10		
	$+Mg^{2+}/P$			10		
DOPC/Chol/DOPE	control	10	6	0	3	2
	$+Ca^{2+}$	10	15	10		
	$+Ca^{2+}/P$	90	77	87	84	86
DOPC/DOPE	control	8	6	2	3	4
	$+Ca^{2+}$	1	6	9		
	$+Ca^{2+}/P$	23	14	64	27	30

[a] Donor vesicles (2 μg) containing trace amounts of ^{125}I-PE and the indicated carrier and fluorescent lipid analogues were treated as described in Figure 1, except that, after the 3-min incubation period, 1 mL of buffer was added and an aliquot was removed for counting. The suspension was then centrifuged for 5 min at 10000*g*, and an aliquot of the supernatant was also counted (see Materials and Methods). [b] The carrier lipid ratios for DOPC/Chol/DOPE and DOPC/DOPE were 40/50/10 and 90/10, respectively. [c] All the fluorescent lipids were 1 mol % with respect to the carrier lipids.

se is not responsible for the inhibition of NBD-PS transfer. This is suggested from the observation that although Ca^{2+}/phosphate effectively aggregated TLE and DOPC/Chol/DOPE vesicles irrespective of the NBD-analogue present, this treatment resulted only in the specific inhibition of NBD-PS transfer (see Table III). Thus, besides the Ca^{2+}/phosphate-induced aggregation of the bulk carrier lipids (TLE and DOPC/Chol/DOPE vesicles) it would appear that Ca^{2+}/phosphate also binds to NBD-PS, resulting in its inability to transfer under appropriate conditions. Indeed, the ability of those vesicles containing "nonaggregating" carrier lipids to precipitate was always enhanced when NBD-PS was included (Table V), albeit without significant inhibition of transfer (Table III). This can be seen from the data presented in Table V, which show that the addition of Ca^{2+}/phosphate to DOPC or DOPC/DOPE vesicles (containing NBD-PS) resulted in specific and dramatic increases in vesicle aggregation from 6 to 16% and from 23 to 64%, respectively. Finally, Mg^{2+} alone or Mg^{2+} in combination with phosphate did not result in vesicle precipitation nor in any decrease in the transfer rate of NBD-PS (see Table III). From these results we conclude that the inhibition of NBD-PS transfer occurs most probably as a result of the intravesicular interaction of Ca^{2+}/phosphate with PS to other membrane components, and not because of Ca^{2+}/phosphate-mediated intervesicular cross-linking (see Discussion).

Discussion

Using previously described techniques of resonance energy transfer (Struck et al., 1981) for determining the transfer rate of fluorescent lipid analogues between vesicles (Nichols & Pagano, 1982, 1983), we show here that NBD-PS can undergo a calcium- and phosphate-mediated and PE- and cholesterol-dependent immobilization in synthetic bilayer membranes. This conclusion is based on the observation that the transfer of NBD-PS, in contrast to other neutral (NBD-PC, NBD-PE, and NBD-DG) or negatively charged (NBD-PG, NBD-PA, and NBD-COOH) NBD analogues, is significantly reduced upon the addition of Ca^{2+} and phosphate to carrier vesicles of appropriate lipid composition. Thus, Ca^{2+}/phosphate inhibition of NBD-PS transfer occurs only when the probe is incorporated into TLE or DOPC/Chol/PE vesicles (see Table IV). Because of the nature of the assay, however, which is based on the absence of a time-dependent increase in NBD fluorescence (escape from rhodamine-mediated quenching), it is conceivable that our results could be interpreted as being due to other "indirect" Ca^{2+}/phosphate-mediated processes such as (1) lateral phase separations inducing a concomitant NBD self-quenching, (2) vesicle–vesicle fusion, and/or (3) vesicle–vesicle aggregation.

Since it has been shown that the fluorescent yield of N-NBD-PE is a sensitive indicator of lateral phase separations in synthetic vesicle systems (Hoekstra, 1982a,b) and is also sensitive to Ca^{2+}-promoted changes in quantum yield (Morris et al., 1985), we investigated whether such a phenomenon was occurring in this system. Experiments designed to test for this possibility (Figure 4) revealed essentially the same relative alterations in the quantum yield of NBD-PS in the absence or presence of Ca^{2+}/phosphate irrespective to the probe concentration or whether DOPC or TLE was used as a carrier lipid. Interestingly, the "inherent" quantum yield (as assessed at infinite probe concentration) of NBD-PS was significantly lower in TLE than in DOPC, a result remarkably similar to our previous observations on the quantum yield of NBD-PS in intact erythrocytes (Tanaka & Schroit, 1983). Although it is difficult to rule out whether the apparent "a priori" quenching of the initial vesicle population (vesicles with and without EDTA[3]) represents some degree of minimal self-association of NBD-PS, it is clear that the addition of Ca^{2+}/phosphate to the vesicles induces essentially the same relative degree of quenching in both DOPC and TLE, systems where transfer is relatively unaffected and inhibited, respectively (Figure 1). Since alterations in NBD-PS packing (as assessed by relative fluorescence) are most sensitive near concentrations of 1 mol % (Tanaka & Schroit, 1983), it would appear that relatively large complexes specific to the TLE system are not being induced. Indeed, it has been shown that vesicles containing high concentrations of NBD-PS (assumed to be analogous to massive NBD-PS self-association) result in quenching factors of 50–100-fold (Tanaka & Schroit, 1983). Furthermore, it appears that this phenomenon is highly specific for PS since the transfer of NBD-COOH is unaffected by Ca^{2+}/phosphate, although this PS analogue binds Ca^{2+} and is "fusogenic" (Nayar & Schroit, 1985). These results suggest, therefore, that Ca^{2+}/phosphate-induced, TLE-dependent phase separations and potential hexagonal H_{II} phase formation are probably not occurring in this system. Further evidence in support of this conclusion is that the ability to inhibit the transfer of NBD-PS in the DOPC/Chol/PE systems seemed to be independent of the amount of NBD-PS used (1–4 mol %; results not shown) and was independent of the acyl-chain composition of the PE employed (Table IV). Thus, DOPE, DPPE, and egg PE were equally effective in facilitating the Ca^{2+}/phosphate-induced inhibition of NBD-PS transfer and support the notion that, in multicompartment membrane systems, Ca^{2+}-induced phase separations of PS are not readily achievable (Tilcock et al., 1984).

Experiments designed to determine whether homotypic TLE vesicle fusion was occurring in the presence of Ca^{2+}/phosphate showed that extensive fusion did not occur. This may be somewhat surprising since it has been previously shown that phosphate reduces the Ca^{2+} threshold required to induce fusion

[3] The relative fluorescence of NBD-labeled lipids is unaffected by buffer composition in the presence of Triton X-100 (unpublished observations).

of PS-containing vesicles (Fraley et al., 1980) and RBC ghosts (Hoekstra et al., 1985). However, Hoekstra et al. (1985) have recently shown that the potential for Ca^{2+} and phosphate to induce fusion of RBC ghosts is extremely sensitive to the Ca^{2+} and phosphate concentrations and required at least 7 mM phosphate, while Ca^{2+} above 2 mM was inhibitory. Therefore, if one assumes that the membrane components responsible for RBC ghost fusion (the lipid component) are similar to those found in our TLE extracts, then fusion would not have been expected to occur since our routine Ca^{2+} and phosphate concentrations were below (3.3 mM) and above (5.0 mM) those required to facilitate fusion.

It is well-known that at appropriate ion concentrations the mixing of Ca^{2+} and phosphate results in a variety of Ca^{2+}/phosphate complexes that range from amorphous Ca^{2+}/phosphate, to octacalcium/phosphate, and finally to stable precipitates of hydroxyapatite (Meyer & Eanes, 1978). Clearly, our results show a requirement for both ions; however, it is noteworthy that preformed Ca^{2+}/phosphate complexes were significantly reduced in their capability of inhibiting NBD-PS transfer (Table I). In contrast with other systems (Fraley et al., 1980; Hoekstra et al., 1985), the order of addition of Ca^{2+} and phosphate did not dramatically affect the ability to inhibit NBD-PS transfer. It seems, therefore, that separate interactions of Ca^{2+} and phosphate with different vesicle lipid components prior to Ca^{2+}/phosphate complexing are required to inhibit transfer. It is difficult to speculate about the Ca^{2+}/phosphate phases needed to inhibit NBD-PS transfer although, clearly, the formation of these complexes after interaction of the ions at the vesicle surface is important. This was concluded from the results which showed that acidic buffers abrogated the inhibition of NBD-PS transfer (Figure 3b), which is also known to inhibit the formation of Ca^{2+}/phosphate complexes (Cotmore et al., 1971; Fraley et al., 1980), and by the immediate and complete resumption in the transfer of NBD-PS upon the addition of EDTA (Figure 3b).

Interestingly, those conditions that resulted in inhibition of NBD-PS transfer were invariably accompanied by a concomitant aggregation of the donor vesicles.[4] This result might indicate that Ca^{2+}/phosphate forms a "trans" (intervesicle) cross-linking via PS, which results in its inability to transfer. However, control experiments revealed that donor vesicle lipid compositions containing NBD-PS, but not amenable to Ca^{2+}/phosphate-induced inhibition of vesicle transfer (DOPC carrier; Tables III and IV), also aggregated (Table V). Indeed, the ability of those vesicles containing "nonaggregating" carrier lipids to precipitate was always enhanced when NBD-PS was included (Table V), albeit without significant inhibition of transfer (Table III). This can be seen from the data presented in Table V, which show that the addition of Ca^{2+}/phosphate to DOPC or DOPC/DOPE vesicles (containing NBD-PS) resulted in specific and dramatic increases in vesicle aggregation from 6 to 16% and from 23 to 64%, respectively. In these systems "trans" PS–Ca^{2+}/phosphate–PS complexes were most certainly formed (resulting in precipitable aggregates), suggesting that trans complex formation per se was not responsible for the inhibition of NBD-PS transfer. It would appear then that Ca^{2+}/phosphate binding in a "cis" complex (intravesicle) to PS and other lipids, but not PS–Ca^{2+}/phosphate–PS, might be responsible for this phenomenon. This is based on the results which show that inhibition of NBD-PS transfer occurs in the absence of "endogenous" PS present in the TLE extracts but instead requires cholesterol and PE. Certainly, NBD-PS complexing is not occurring here, since if it were, some degree of NBD-PS self-quenching (i.e., domain formation with a concomitant increase in "local" probe concentration) should have been observed. We believe, therefore, that the inhibition of NBD-PS transfer is a result of the immobilization of NBD-PS via Ca^{2+}/phosphate to cholesterol and PE. This, in turn, would cause the NBD-PS to be motionally restricted because of the formation of Ca^{2+}/phosphate domains that results in a significant reduction in the ability of the probe to undergo vesicle–vesicle transfer.

It is difficult to relate the results obtained from the studies presented here to more physiological systems, although it is conceivable that similar Ca^{2+}-mediated processes might be operative in intact erythrocytes. Indeed, we have previously shown that both short (C6) and long (C12) acyl chain labeled NBD-PS become stably incorporated in the membranes of mouse (Tanaka & Schroit, 1983; Schroit et al., 1985) and human (Schwartz et al., 1985) RBC, as opposed to other similarly labeled lipids. It is conceivable that this stability could be envisioned as having been due to a specific translocation of the PS to the inner leaflet of the RBC (Seigneuret & Devaux, 1984). However, contrary to this supposition, the accessibility of NBD-PS to trinitrophenylation (under nonpenetrating conditions) showed that a substantial fraction of the probe was, indeed, in the outer leaflet of the RBC (Schroit et al., 1985). Combined with these previous results, the data presented here do suggest that Ca^{2+} and phosphate can effectively immobilize PS in artificial membrane systems, a process that requires the presence of PE. It is possible, therefore, that such a mechanism might also be operative in intact RBC, resulting in the immobilization of NBD-PS—a property characterized by the inability of NBD-PS to be removed from the cells (Tanaka & Schroit, 1983; Schroit et al., 1985).

Although one might agree that a specific mechanism for the translocation of aminophospholipids across membrane bilayers might exist, alternative mechanisms are also feasible. Since the combined action of Ca^{2+} and phosphate can induce a reorientation of membrane proteins (Carraway et al., 1975; Haest & Deuticke, 1976; Mohandas et al., 1982), it is tempting to speculate that in RBC these lipids might be physically restrained to the inner leaflet via Ca^{2+}/phosphate-mediated processes and, under appropriate conditions, Ca^{2+} might be sequestered away from these lipids, allowing them to attain a partial equilibrium between the bilayers. A possible example of this phenomenon might be in the very recent observations of Lew et al. (1985), who have shown that irreversible sickle cells (ISC) possess intracellular inside-out vesicles in which nearly all of the measurable Ca^{2+} is within these vesicles in the form of precipitates with phosphate. Although these authors relate their findings to the initiation of dehydration and promotion of the sickling process, we find it intriguing that ISC express significant amounts of PS in their outer leaflets (Chiu et al., 1979, 1981) as opposed to the strict asymmetric distribution of PS in normal RBC (Gordesky et al., 1975).

In conclusion, our results, together with the above observations, suggest that Ca^{2+}/phosphate complexes might play an important role in the maintenance of membrane asymmetry by immobilizing PS in a membrane bilayer. Studies to try to validate this possibility, using both artificial membrane systems and intact RBC, are currently in progress.

ACKNOWLEDGMENTS

We express our gratitude to Dr. J. Wylie Nichols for his helpful discussions and to John Madsen, whose expert technical

[4] The inclusion of acceptor vesicles (1 mL; 200 μg of lipid) instead of buffer in the aggregation experiments had absolutely no effect on the extent of Ca^{2+}/phosphate-induced vesicle precipitation.

assistance is gratefully acknowledged.

Registry No. Chol, 57-88-5; DOPC, 10015-85-7; DOPE, 2462-63-7; DPPE, 3026-45-7; Ca, 7440-70-2; PO_4^{3-}, 14265-44-2.

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