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A Membrane-Bound Fluorescent Probe to Detect Phospholipid Vesicle-Cell Fusion

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Summary. Pyrenesulfonylphosphatidylethanolamine has been incorporated into sonicated phospholipid vesicles to provide a fluorescent signal from a membrane-bound probe whose spectrum is sensitive to the local concentration of dye molecules. When vesicle material was taken up by viable mouse splenocytes, the disappearance of the pyrene excimer fluorescence emission peak that accompanied dilution of the vesicle membrane lipid could be quantitated. One can thus measure, by a simple and rapid procedure, a new parameter which is related to the extent of vesicle-cell fusion and which is independent of the transfer of aqueous vesicle contents to the cell cytoplasm.

The interaction of phospholipid vesicles (PLV)[1] with living cells is a recently developed area of considerable interest, both for the fundamental insights to be gained into natural phenomena and for potential practical applications. In the former category are found basic investigations of membrane fusion for relation to endocytosis, cell division etc., along with studies using PLV fusion to modify cells by internal administration of compounds of interest. In the laboratory, vesicles are proving useful for handling and purifying cell surface proteins [1, 10]. The potential clinical applications which have been discussed most often center around the possible use of PLV as an *in vivo* drug delivery technique [2, 15].

In any experimental system interactions between vesicles and cells can be of several types, and distinguishing between these is frequently crucial to the interpretation of results. The interactions may result in: (1) stable adsorption of vesicles with no exchange of cell and PLV material in either the internal or membrane compartments; (2) mixing of internal aqueous phase material only, in particular by transfer of vesicle contents to the cell cytoplasm; (3) mixing of membrane consituents only, such as by lipid exchange; (4) complete fusion, resulting in uniform spreading of PLV lipids in cell membrane and transfer of aqueous contents into the cell cytoplasm; or (5) endocytosis in which vesicles remain intact within the cell without significant mixing of aqueous or lipid bilayer phases. Which of these will predominate in a given system depends in a complex way on the properties of the vesicles and cells, as well as the suspension medium, the temperature, and the duration of incubation.

A number of studies by various authors reporting uptake of vesicle material by cells have been reviewed in [8] and [9]. As these authors have emphasized, however, multiple independent assays are required and caution must be exercised in attributing observed uptake phenomena to fusion events [4, 8, 9]. No single type of measurement can eliminate all other possible mechanisms. For example, the detection of vesicle membrane-bound antigens on the cell surface [6] does not distinguish between fusion and adsorption, and the detection of radioisotopes from the vesicle interior in the cell cytoplasm does not reliably distinguish between fusion and endocytosis.

In this regard, one of the most useful assays is the fluorescence technique of Weinstein, et al. [14], which is now widely employed. These workers placed a concentration-sensitive fluorescent probe in the vesicle interior so that they not only could observe the transfer to the cytoplasm of a cell, but, in addition,

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[1] *Abbreviations used:* 6-CF = 6-carboxyfluorescein; HBSS = Hanks Balanced Salt Solution; DMPC = dimyristoylphosphatidylcholine; DOPC = dioleoyl-phosphatidylcholine; DPPC = dipalmitoylphosphatidylcholine; EYL = egg yolk lecithin; PE = phosphatidylethanolamine; PEG = polyethylene glycol; PLV = phospholipid vesicle; PSPE = pyrenesulfonylphosphatidylethanolamine.

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see if the probe had been diluted in the process. The fluorophore they employed was 6-carboxyfluorescein which was placed in the vesicles at a strongly self-quenching concentration (200 mM). The many thousandfold dilution of the dye upon release into the cell resulted in a greater than 30-fold increase in 6-CF fluorescence. The resulting cytoplasmic fluorescence was visible under the fluorescence microscope or by flow cytofluorometry [14].

Since the soluble fluorescein technique leaves unanswered the question of the fate of the vesicle membrane components after interaction with the cell, a similar dilution-sensitive technique employing a membrane-bound probe would provide extremely useful additional information. It would indicate whether cell membranes become widely labeled after interaction with PLV or whether vesicle lipids remain sequestered, either at the cell surface or in internal endocytic vacuoles. For this purpose a fluorescent probe is again the physical method of choice due to the rapidity with which measurements can be made without fixing or other special preparation of samples.

Pyrene is a fluorophore which has been employed in membrane bilayers and whose fluorescence emission spectrum is known to be sensitive to the concentration of dye [11]. The concentration dependence arises because an optically excited pyrene molecule can decay not only by the usual single-molecule radiative and non-radiative routes, but also by an interaction with a nearby unexcited molecule to form an excited state dimer (excimer). The extent of such excimer formation naturally depends on the local concentration of available ground-state molecules. Furthermore, the excimer species can be distinguished in the fluorescence emission spectrum since excimer radiative decay occurs in a broad peak, at longer wavelengths, which is well separated from the sharp monomer emission peaks. Excimer formation, and the concomitant quenching of monomer fluorescence, depends on the concentration of dye in the lipid bilayer and, in addition, on the fluid nature of the membrane which allows lateral diffusion of dye molecules [7, 12].

Although pyrene itself is quite hydrophobic and partitions well into membranes in suspension, we have chosen to use a commercially available phosphatidylethanolamine derivative, since this convenient probe will have hydrophobicity and lateral mobility most similar to the other membrane components of the vesicles we are interested in fusing. The compound is N-(1-pyrenesulfonyl)-dipalmitoyl-L-α-phosphatidylethanolamine (PSPE). The structure and fluorescence spectra are shown in Fig. 1. The similar use of excimer emission from a pyrene-cholesterol derivative has recently been reported by Via et al. [13] for monitoring vesicle-vesicle fusion.

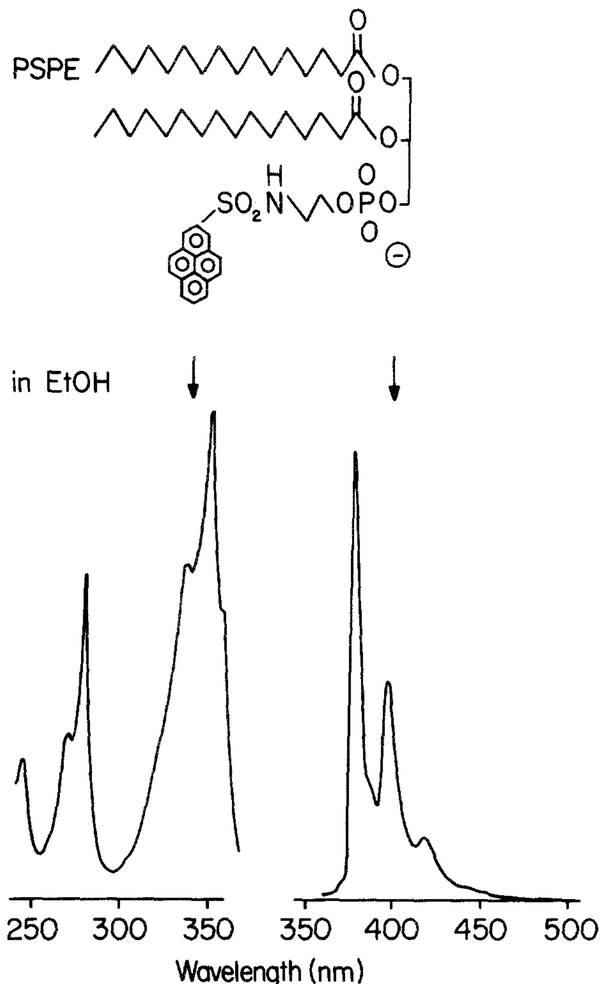

Fig. 1. Structure of pyrenesulfonylphosphatidylethanolamine and the excitation and emission spectra in dilute solution in ethanol. The arrows indicate the exciting wavelength for the emission spectrum and the monitored wavelength for the excitation spectrum. In both cases the slit width for the wavelength being scanned was 1 nm

Materials and Methods

Vesicles and Dye

The PSPE dye was synthesized by Molecular Probes, (Plano, Tex.) from pyrenesulfonilic acid and synthetic (fully saturated) dipalmitoylphosphatidylethanolamine (Sigma Chemical Co.). Vesicles containing PSPE were prepared by sonication using synthetic phosphatidylcholine (DPPC, DMPC, DOPC) or natural EYL from Sigma (St. Louis, Mo.). The mixture was dissolved in approximately 0.5 ml chloroform, which was then evaporated under a stream of dry nitrogen and approximately 1 ml of buffer was added (Hanks balanced salt solution with Ca^{++} and Mg^{++}, Microbiological Associates, Bethesda, Md.). The mixture (about 5 mg lipid per ml) was vortexed vigorously to suspend the hydrophobic constituents, transferred to a plastic test tube, and sonicated until no further clarification could be observed. This required about 3–5 min (Branson model W185 sonifier with microprobe attachment).

Cells and Incubations

Spleen cells were obtained from unimmunized BALB/c mice by gently disrupting the spleen tissue between frosted glass surfaces. During preparation the buffer was augmented with 5% heat-inactivated fetal calf serum (Gibco, Grand Island, N.Y.). The resulting cell suspension was washed by centrifugation and red cells were lysed by a 2-min exposure to Tris-buffered NH_4Cl or hypotonic shock (10–30 sec in buffer diluted with distilled water). The viability of the cell suspension was assessed by the Trypan blue dye exclusion test. Cell counts were performed promptly after addition of the dye to avoid artificially high viability assessments due to the disappearance of dead cells by lysis following dye uptake.

Cells were incubated with vesicles in serum free buffer (HBSS + Ca^{++} + Mg^{++}) at 37 °C for 30 min with 2 mg vesicles + 10^7 cells in 2 ml unless otherwise noted. After incubation the cells were washed twice, without change of tubes, in ice cold HBSS and kept on the ice until the fluorescence spectrum was measured.

Samples of cells + associated vesicular material, after washing and determination of fluorescence, were treated with trypsin or with polyethylene glycol (PEG). Trypsin treatments were carried out as described by Huang et al. [5] (0.01% enzyme for 10 min at 37 °C). The PEG treatment was derived from Milstein's protocol for cell hybridization [3]. The principal change which was made for these studies was that cell densities were kept low to avoid cell-cell fusion events. Pelleted cells (approximately 10^7) were suspended in 0.4 ml buffer (HBSS with Ca^{++} and Mg^{++}) and 1 ml of 50% PEG (wt/vol) was added at room temperature and mixed. The suspension was then promptly diluted to 15 ml with buffer, the cells were washed by centrifugation and then kept on ice.

Fluorescence

Spectra were obtained on a Perkin-Elmer 650-10S spectrofluorometer with a thermostatted cuvette holder. Unless otherwise noted, the emission of PSPE was scanned using a 1 nm slit width while exciting the sample at 340 nm with an excitation slit width of 3–10 nm.

Results

The spectra of the pyrene-PE compound (PSPE) showed the same dependence upon concentration as was seen by Vanderkooi and Callis for pyrene in sonicated PLV [11]. The spectrum of the dilute probe, shown in Fig. 1, is similar to that of pyrene, with some modifications due to the addition of the sulfonyl group to the conjugated pyrene ring structure. In an aqueous system with PSPE incorporated into membrane vesicles, the ratio of PSPE to phosphatidylcholine determined the frequency of collisions between dye molecules and thus the shape of the emission spectrum. A series of spectra for samples containing from 10 to 100% PSPE in DMPC is shown in Fig. 2.

The effect of extreme dilution due to the addition of nonionic detergent to a vesicle sample was to

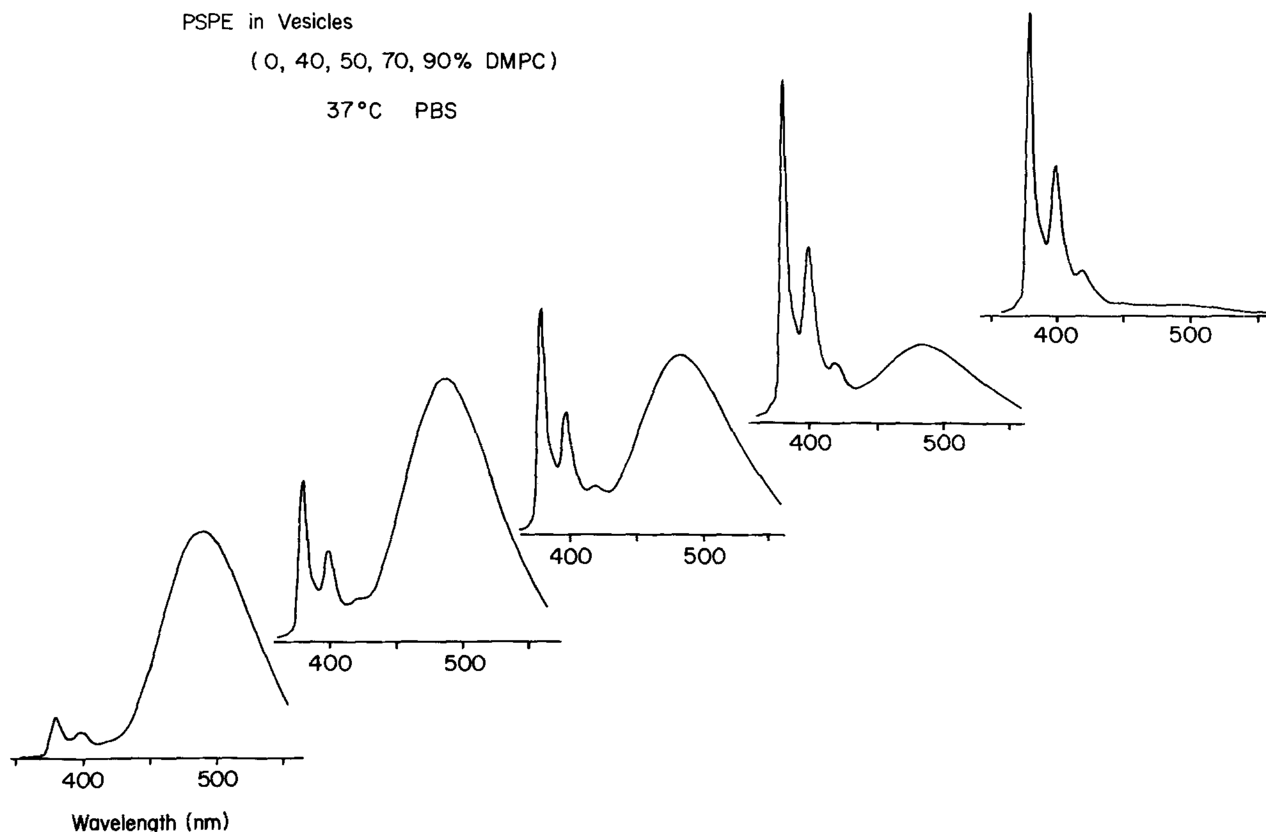

Fig. 2. Pyrene-sulfonyl-PE incorporated into dimyristoyl-PC vesicles at different ratios from 10–100% PSPE

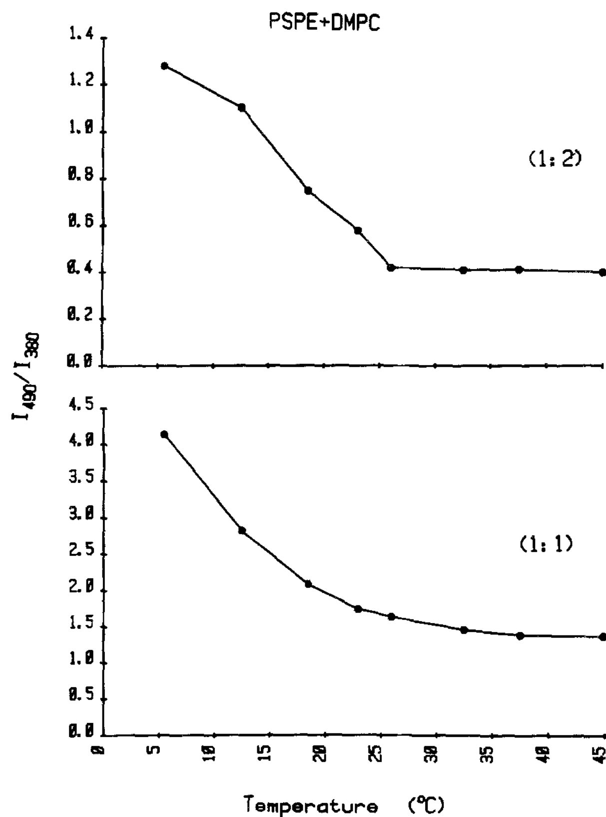

Fig. 3. Temperature dependence of the ratio of excimer (490 nm) to monomer (380 nm) peak heights in the spectra of PSPE+DMPC vesicles showing the influence of the DMPC phase transition

Table 1.

Lipid component	DMPC	DOPC	DPPC	EYL
Lipid/PSPE ratio	2:1	2:1	4.5:1	6:1
Relative excimer Fluorescence[a] in:				
Original vesicles	0.97	0.77	0.80	0.40
Cells after incubation[b]:				
At 4 °C	0.58	0.57	0.56	0.35
At 37 °C	0.46	0.50	0.90	0.22
At 4 °C+trypsin[c]	0.45	0.30	0.33	0.27
At 37 °C+trypsin[c]	0.33	0.20	0.53	0.22

[a] Relative excimer fluorescence is defined as the ratio (emission peak height at 490 nm) ÷ (peak height at 380 nm).

[b] Incubations were for 1 h in HBSS with Ca^{++} and Mg^{++}, at the temperature indicated, with 10^7 splenocytes and 1 mg sonicated vesicles in 2 ml.

[c] Cells previously incubated and measured were then trypsin treated (*see* Methods), washed, and emission spectra remeasured.

cause a dramatic decrease in the excimer peak relative to the monomer. When a similar measurement was performed by adding detergent to a sample of pure PSPE, which showed only excimer emission, the resulting monomer spectrum had a peak height 2.1 times the original excimer when it was measured at the same instrument settings (not shown).

The temperature dependence data, shown in Fig. 3, indicate that the PSPE probe in these vesicles is sensitive to the properties of the lipid environment

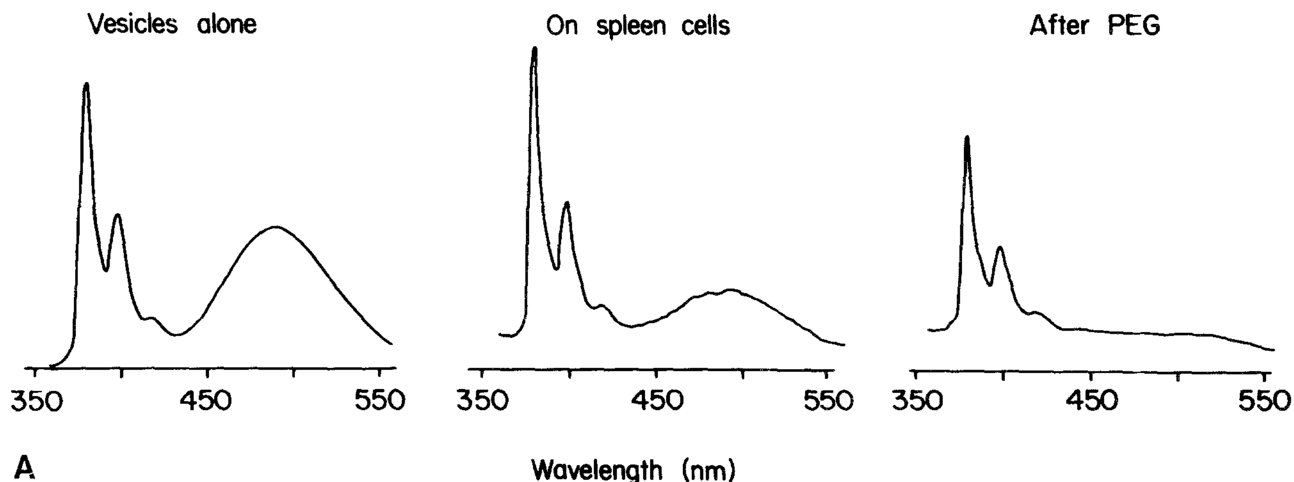

Fig. 4. Emission spectra of PSPE in dioleoyl-PC vesicles (A), dimyristoyl-PC vesicles (B), and dipalmitoyl-PC vesicles (C). The spectrum on the left in each set is the original vesicle preparation. The spectrum in the center is of washed cells after incubation with vesicles, and the spectrum on the right is from the same cells after treatment with polyethylene glycol to enhance fusion with adsorbed vesicles

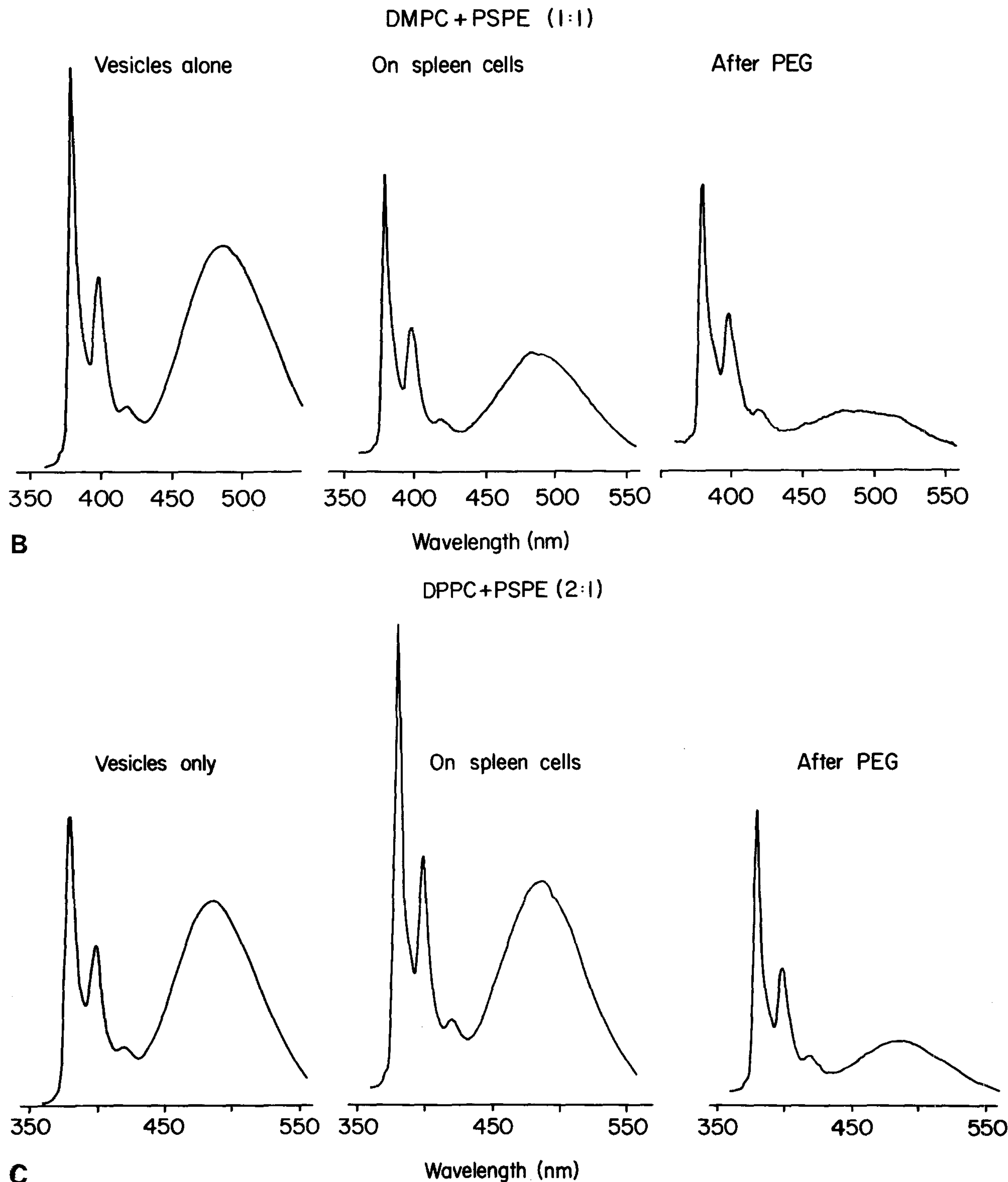

Fig. 4.

in which it is placed. Even at the relatively high levels of PSPE required to observe the excimer fluorescence, the phase transition temperature for the DMPC component (22 °C) can be seen in the graph of relative excimer emission *vs.* temperature.

The inflection is sharper in the more dilute system (1:2 by weight, PSPE/DMPC) while total excimer fluorescence was greater in the case of vesicles which were 1:1 (wt/wt). Similar measurements on vesicles composed of DPPC+PSPE (not shown) showed a

more pronounced overall dependence on temperature, but with no indication of a change in properties at any temperature in the range 5–37 °C. This was consistent with the higher transition temperature of DPPC.

Vesicles consisting of PSPE sonicated with different lipids were incubated with mouse splenocytes in experiments which showed that PSPE excimer fluorescence was sensitive to vesicle-cell interactions. In every case (>50 experiments), the incubation of viable splenocytes with sonicated vesicles in serum-free buffer resulted in a decrease of relative excimer fluorescence in the spectra of material binding to the cells, as compared with the original PLV. The extent of excimer loss depended on the lipid chosen and the temperature of incubation but was relatively insensitive to the duration of incubation. We also saw little dependence upon the ratio of PSPE to lipid in the vesicles.

Four typical experiments are shown in Table 1, which presents the ratio of excimer to monomer peak heights of four vesicle samples and for the material adherent to washed cells after incubation with each, both at 4° and at 37 °C. In most cases the extent of the disappearance of excimer fluorescence suggested a greater degree of fusion at 37° than at 4°, as might be expected [5]. Cell viabilities remained greater than 70%, as measured by Trypan Blue exclusion.

Cells that had been incubated with vesicles and washed twice were also examined under the fluorescence microscope. Visible excimer emission was observed on all or nearly all the cells of each sample with little or none arising from debris. No subpopulation of cells appeared to dominate the fluorescence emission. The surfaces of cells incubated at 37° were more uniformly fluorescent while those incubated at 4° showed a more patchy appearance.

The cells were then treated with trypsin to remove adsorbed vesicles. This resulted in a further diminution of relative excimer fluorescence (Table 1). About 60–70% of the remaining dye on the cells in each case appeared to be in a dilute form since the relative excimer fluorescence was only 30–40% that of the original vesicles.

A second set of experiments which linked excimer disappearance with a probable fusion phenomenon involved a treatment with 50% polyethylene glycol of lymphocytes which had been pre-incubated with fluorescent vesicles and washed by centrifugation. The spectra in Fig. 4 clearly show a loss of relative excimer fluorescence, which we attribute to a dilution of the PSPE probe, possibly resulting from vesicle-cell fusion events. The 30–50% decrease which followed uptake by cells can be seen in the figure by comparing the first and second spectra in each set. After treatment with PEG (third spectrum) the excimer was further diminished to 30–40% of the relative height observed in the original vesicles.

The available evidence indicates that the observed fluorescence in cells after incubation with PSPE vesicles and washing was firmly attached to the cells, since the excimer emission that was observed by fluorescence microscopy arose from cells only, and much of the signal remained associated with the cells through later treatments with PEG or trypsin. In addition, it was noted in a separate experiment (data not shown) that the total PSPE uptake by a fixed number of cells (5×10^6) was independent of the amount of vesicle material in the incubation medium (from 0.05 to 0.50 mg/ml).

A quantitative estimate of the amount of vesicular material which was associated with the cells after incubation could be obtained from the magnitudes of the fluorescence signals. From the sensitivity settings of the instrument for the spectra in Fig. 4 we infer that the cell uptake of vesicles under our incubation conditions came to approximately 10^{-8} g/10^6 cells for DPPC+PSPE vesicles (about 6×10^6 molecules/cell). The values for DOPC appeared to be fivefold greater and the value for DMPC about fivefold less.

Discussion

The spectra in Figs. 1 and 2 above show that pyrene-conjugated phosphatidylethanolamine can perform as an indicator of dilution of vesicle membrane material. In addition, the quantitative information one obtains by fluorescence spectroscopy allows estimates to be made of the fraction of vesicles which have fused with cell surface membranes to give rise to the observed difference the spectra of free vesicles and washed cells after incubation with PLV.

Assuming that vesicles which interact with cells undergo either stable adsorption (spectrum unchanged) or fusion with complete loss of excimer emission, we can mathematically estimate from the spectra the proportion involved in each mechanism. Let R and R' be the relative excimer peak heights before and after interaction with cells, respectively, f be the fraction of the original vesicles remaining intact, and d be the ratio of the monomer and excimer heights for PSPE which undergoes dilution from purely excimer to purely monomer emission (equal to 2.1 by

our measurement). Then one can obtain the following formula relating the quantities:

$$R' = \frac{\text{new excimer height}}{\text{new monomer height}} = \frac{fR}{f+(1-f)d}. \quad (1)$$

If this formula is solved for f and applied to the spectra shown in Fig. 4 we obtain values of approximately 70%, 83%, and 80% for the percentage of intact vesicles on the surfaces of cells incubated with DOPC, DMPC and DPPC vesicles, respectively. After the treatment with PEG, the percentage of intact PLV dropped to 35–55% in the case of DOPC, 45–55% in the case of DMPC, and 48% for DPPC. The spreads in estimated values in the first two cases reflect some uncertainty in the baselines, due to light scattering, in the spectra of Figs. 4*a* and *b*.

The assumption that PSPE from vesicles which have fused will contribute no excimer can be justified from the small amounts of vesicle uptake. Only about 5×10^{-8} g of PLV material appears to be associated with the 5×10^{6} washed cells after incubation. If we estimate the mass of the plasma membranes to be approximately 5×10^{-6} g, then vesicles which have fused should have experienced at least a 100-fold dilution. It is clear from the spectra in Fig. 2 that the excimer peak should completely disappear under these conditions.

It is of interest to note that using our incubation conditions the fraction of cell-associated vesicle material which appeared to be fused rather than adsorbed, as estimated from the PSPE fluorescence, rarely exceeded 50%, even after treatment with trypsin or PEG. The fluorescent probe suggests that there may have been a sizable population of vesicles which firmly adhered to the cell or was endocytosed in our procedure. These resisted removal by trypsin but nevertheless have apparently not fused with the cell plasma membrane. The spectrofluorometric assay is well suited for this quantitation since it is not strongly biased toward the detection of either the fused or unfused PLV. It thus complements the fluorescein release assay [14] which detects vesicle fusion by a 30-fold increase in fluorescence, without the potential for spectral discrimination between emissions from the concentrated and the dilute dye.

Since the PSPE excimer emission is in a region to which the eye is sensitive, adsorbed vesicles on the surfaces of cells can be easily visualized under the fluorescence microscope using ultraviolet illumination. The monomer peaks are of too short a wavelength to be detectable in this way, but it has proven possible to qualitatively observe the dilution of vesicle membrane material by the disappearance of the visible blue-green excimer fluorescence. Conversely, incubation conditions which resulted in little or no loss of the excimer spectral peak yielded cells which exhibited clear and non-uniform blue-green surface fluorescence. In either case, it was possible to check directly that little or no excimer emission arose from debris or from any minor population of highly fluorescent cells.

Fluorescence instrumentation is sufficiently sensitive that the PSPE probe could be used at very low concentrations, which would show a monomer emission spectrum only, to follow PLV uptake by cells. However, higher concentrations of fluorophore are required if one wishes to obtain excimer fluorescence in the PLV and look for the dilution phenomenon. Since vesicles must be composed of 10–50% PSPE for this technique, experiments which vary the lipid in the vesicle cannot readily be compared with results that obtain with pure phosphatidylcholine vesicles. The relatively large amounts of the negatively charged probe may also account for the relatively low uptake of labelled PLV by cells in these experiments.

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