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Preparation of Lucifer Yellow Fluorescent Liposomes: A Method for Cells' Membrane Labeling[1]

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This report describes a method to conjugate lucifer yellow to the external surface of liposomes. The heterobifunctional cross-linking reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate has been used to activate DMPE molecules. The DMPE-dithiopyridine product has been mixed with DMPC to prepare liposome vesicles. These have been reduced by DTT and finally reacted with lucifer yellow-iodoacetamide to produce the fluorescence-labeled vesicles. The quenching of their fluorescence intensity by KI is consistent with fully exposed fluorophores. The decay of the fluorescence intensity of the lipid-bound lucifer yellow is biexponential ($\tau_1 = 7.9$ ns; $\tau_2 = 1.1$ ns), with a relative yield of 0.16. When the fluorescent liposomes are mixed with cells, the lucifer yellow-DMPE derivative is transferred. Boar spermatozoa and peripheral human blood lymphocytes have been used as cellular models. The extent of incorporation is dependent on the incubation time and temperature. At 36°C, lucifer yellow fluorescence appears in the spermatozoa cells after 10 min of incubation and reaches its maximum at about 60 min. The fluorescent phospholipid derivative seems to incorporate specifically into membrane structures. The highest labeling ratio is observed with integer, scarcely motile, spermatozoa. A poorer labeling yield (≈15%) is found with lymphocytes. Interestingly, photobleaching due to epiillumination of the labeled cells is apparently negligible and cells are clearly visible after irradiation times ranging from several minutes to few hours.

KEY WORDS: Lucifer yellow; liposomes; fluorescent dyes; membranes.

INTRODUCTION

Since its introduction in 1978 [1], the fluorescent dye lucifer yellow (LY) gained the attention of the biologists' community for its photphysical properties. The high quantum yield (QY = 0.21), the large Stokes' shift (ex = 430 nm; em = 530 nm), and its low toxicity [2] represent excellent characteristics for spectroscopical studies in solution and for cell biology fluorescence microscopy investigations. The most frequent application of the LY dyes has been as a polar neuronal tracer. In the earliest studies, in fact, lucifer yellow carbohydrazide (LY-CH) was used to trace the cell bodies of neurons and to reveal their shape [1–6]. Because of its high polarity, LY cannot cross the cell membrane barrier. Thus, once in the cytosol, the dye is trapped within the cell. On the other hand, simple diffusion from the medium cannot be used to bring the dye inside the cell and different mechanical methods must be considered. Some techniques that have been found very effective in neurobiology studies have been presented and reviewed by

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Stewart [2], namely, (i) the intracellular injection of the dye through a micropipette, (ii) the backfilling of the dye through the cut end of a nerve plunged in a tracer solution, and (iii) retrograde axonal transport of lucifer yellow vinyl sulfone (LY-VS) by *in situ* conjugation to proteins. However, these methods are relatively invasive and cannot be used with all types of cells.

More recently, LY-CH has been described to be suitable for monitoring fluid-phase pinocytosis in macrophages and for identifying compartments of the endocytic pathway [7]. Although in most research applications the probe has been dissolved in solution, the covalent labeling of cell membranes with LY dyes would be of practical importance. In this regard, Spiegel *et al.* [8] have described a procedure in which they have taken advantage of LY-CH reactivity for oxidized glycoconjugates to label the cellular surface of thymocytes.

In the present report we present a noninvasive and specific procedure to label cell membranes by LY using liposomes as a vector. LY-iodacetamide is conjugated to thiolated lipid vesicles after their formation. Thus, the procedure can be regarded as a general method for *in situ* conjugation of liposomes with SH-reactive fluorescent compounds. The dye is present as a covalent derivative of dimyristoyl-L-α-phosphatidylethanolamine (DMPE), and after incubation, the fluorescent phospholipid is transferred to cells. The results obtained with spermatozoa cells and lymphocytes are presented.

MATERIALS AND METHODS

Dimyristoyl-L-α-phosphatidylcholine (DMPC), DMPE, dimyristoyl-L-α-phosphatidylserine (DMPS), and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were purchased from Sigma Chemical Co.. Purity of the compounds was verified by TLC on heat-activated silica gel 60 plates. LY-CH potassium salt and LY-iodacetamide were obtained from Molecular Probes, Inc.. Sephadex LH-20, G-50, and Ficoll/Isopaque were from Pharmacia Fine Chemicals and used following the instructions of the supplier. RPMI 1640 was from Seromed, Milan, Italy. Octyl-glucoside was obtained from Fluka and Kl from BDH. Triethylamine (Et_3N), from Merck, was stored in the dark at 4°C. Methanol and chloroform, UVasol, and HPLC grace, were from Merck. Thin-layer chromatography was performed on analytical TLC silica gel 60 aluminum plates, from Merck, and eluted in one dimension using chloroform/methanol/water (65:25:4, v:v) as eluting solvent. TLC spot identification was carried out by phosphorus staining reaction [9] to reveal the presence of phospholipid and by ninhydrin reaction [10] to reveal free NH_2 groups. The concentration of phospholipid was determined from the phosphorus content [11].

DMPE Modification. The heterobifunctional crosslinking reagent SPDP has been used to activate DMPE by introduction of the 2-pyridyl disulfide group as previously described [12, 13]. Briefly, 20 mg of DMPE was dissolved in 5–6 ml of a methanol/chloroform solution (1:1, v/v) containing 20 μl of Et_3N. A twofold molar excess of SPDP was then added, at 25°C, to the organic solution. The reaction of the amino group of DMPE with the *N*-hydroxysuccinimide ester side of the reagent was monitored by the disappearance of the purple, ninhydrin-positive, spot on TLC ($R_f = 0.70$). Typically, formation of DMPE-DTP was complete in 2 h. At this stage, the product migrated close to the front of the eluting solvent on TLC plates; it was positive to the phosphorus staining reagent but negative to ninhydrin. Finally, the dithiopyridyl-containing DMPE was applied to a 1.5 × 25-cm Sephadex LH-20 gel filtration column, equilibrated with methanol at 4°C, to eliminate the *N*-hydroxysuccinimide groups released upon amide bond formation. The lipid-containing fractions (0.8 ml each) eluted with the void volume were pooled and stored in the dark at −20°C. Before use, the purity of DMPE-DTP was always checked on analytical TLC plates.

Vesicle Preparation and Labeling. The product, DMPE-DTP, was mixed with a methanol/chloroform solution (1:1, v/v) containing 5 mg/ml of DMPC at a proportion of 1:10 (DMPE-DTP/DMPC, mol:mol). The mixture was dried under a nitrogen stream and used to prepare the dithiopyridyl-bearing liposomes.

Small unilamellar vesicles (SUV) were prepared by sonication, and large unilamellar vesicles (LUV) by the method of octyl-glucoside dialysis [14], using Tris–HCl 10 m*M*, pH 7.4, 0.137 *M* NaCl as a suspending buffer. The disulfide groups at the liposomes surface were reduced by adding DTT (10 m*M*, 1 h). Then the reducing reagent was eliminated by dialysis against the suspending buffer (4 h). At the end of this step, the liposomes were left to react with the SH-reactive LY derivative, LY-iodoacetamide, for 2 h. The vesicles were separated from the free unreacted dye molecules by passing the lipid dispersion through a 1.5 × 25-cm G-50 Sephadex gel filtration column, at 4°C, and equilibrated with the same solution as the vesicle suspension. The liposomes were eluted near the void volume and presented the typical fluorescence properties of LY. These vesicles (3–4 m*M*, corresponding to an approximate DMPE-LY content of 0.32 m*M*) were used for the spectroscopic studies

and for cell labeling experiments. DMPE-LY liposome concentrations were calculated from the total phosphorus content [11], assuming that all the DMPE-DTP reacted with LY-iodoacetamide.

The same procedures were used to prepare DMPE-LY/DMPC/DMPS (1:7:2, mol/mol) liposomes.

Cell Preparation. The semen of large white boars was generously provided by Semen-Italy, Modena, Italy, and processed within 2 h of collection. Cells were washed three times in 10 m*M* Tris–HCl, pH 7.4, containing 0.137 *M* NaCl, (500*g* for 8 min), and resuspended in the same buffer. Cells were mixed with the fluorescent liposomes by adding 0.5 ml of cells (40×10^7 cells/ml) to an equal volume of a liposome suspension (3–4 m*M*). After incubation, cells were washed in the same buffer reported above and used for microscopy observations. Sperm cell viability and acrosomal membrane integrity were assayed by Trypan blue dye exclusion and the Giemsa reaction [15], respectively.

Human blood was obtained from the blood bank. Blood was layered on a Ficoll/Isopaque gradient and centrifuged at 400*g* for 30 min; lymphocytes were collected and washed three times with PBS at 100*g* for 15 min. Finally, peripheral blood lymphocytes were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640. Cultures were maintained at 37°C overnight in a humidified 5% CO_2 atmosphere to allow the monocytes to adhere to the culture flask. To prepare a typical lymphocyte incubation sample, the cells were removed from the culture flask with the medium and washed twice with PBS. To 0.5 ml of the lymphocyte suspension (1×10^6 cells/ml) an equal volume of the fluorescent vesicles was then added. Cells were washed twice and then analyzed under the fluorescence microscope. Alternatively, lymphocytes were spread on polylysine-coated culture dishes. In this case, 0.5 ml of the liposome suspension was added to the wells after removal of the culture medium.

Observations of control incubation samples, prepared using unlabeled liposomes, were taken in all cases to rule out autofluorescence contributions.

Fluorescence Spectroscopy Measurements. Steady-state fluorescence intensity measurements were performed with a Jasco FP-550 spectrofluorometer, exciting the samples at 430 nm. Excitation and emission band widths of 5 and 10 nm were used, respectively. For fluorescence intensity quenching experiments, stock solutions of KI (4 *M*) were freshly prepared in the presence of $\approx 1 \times 10^{-4}$ *M* $Na_2S_2O_3$ to avoid I_3^- formation [16]. Samples at increasing quencher concentrations were prepared by the addition of small aliquots of KI by means of a syringe. The absorbance observed at 430 nm was always below 0.08 OD. The fluorescence quenching data were analyzed according to the Stern–Volmer equation [16]:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = \frac{\tau_0}{\tau} \tag{1}$$

where F_O and τ_0 are the fluorescence intensity and the lifetime measured in the absence of the quencher, respectively, and F and τ are the fluorescence intensity and the lifetime measured in the presence of the quencher, respectively. K_{sv} is the Stern–Volmer constant, which is related to the bimolecular quenching rate, k_q, by the relationship $K_{sv} = k_q\tau_0$.

Steady-state fluorescence anisotropy measurements were performed using a Polacoat dichroic polarizer and analyzer (Ealing Electroptics, UK) placed in the excitation and emission paths, respectively. The samples were excited at 430 nm and emission was observed at 530 nm, with 10-nm excitation and emission band widths. The steady-state anisotropy, $\langle r \rangle$, was calculated according to the following relationship:

$$\langle r \rangle = \frac{I_{vv}(G) - I_{hv}}{I_{vv}(G) + 2I_{hv}} \tag{2}$$

where I_{vv} and I_{hv} represent the parallel and perpendicular components of the fluorescence intensity, respectively. G is the "grating" factor to correct for the polarized light instrumentation bias [17].

Time-resolved fluorescence measurements were performed by the single-photon counting method using a fluorometer optical configuration equipped with the typical NIM electronic modules (EG&G Ortec, TN) and a thyratron-gated nitrogen flash lamp (Model F199, Edinburgh Instruments, Edinburgh, Scotland) as the excitation source [17]. Fluorescence decay experimental data were carried out under "magic-angle" conditions [17]. Data analysis was obtained by a nonlinear least-squares procedure, assuming that the fluorescence intensity decay is well described by a sum of exponential terms of the following form [17]:

$$I(t) = \sum \alpha_i \cdot e^{-t/\tau_i} \tag{3}$$

where α_i are the intensity amplitudes associated with each lifetime component, τ_i. The plot of the weighted residuals, their autocorrelation function, and the χ^2 value were the statistical parameters used to judge the quality of the fitting procedures.

Fluorescence microscopy measurements were performed with a Carl Zeiss Axioplan fluorescence microscope using a broad-bandpass fluorescein filter.

RESULTS

Labeled Vesicle Preparation. In the present work both SUV and LUV fluorescent vesicles were prepared. No significant differences were found between preparations, as far as the formation, the labeling, and the chromatography separation of the vesicles are concerned.

In Fig. 1, the typical elution profile of the vesicles passed through a Sephadex G-50 column, after labeling with LY-iodoacetamide, is presented. The vesicles are eluted near the void volume and present intense fluorescence at 530 nm. The fluorescence elution profile overlaps with the phosphorus content of each fraction. As shown in Fig. 2, the excitation and emission spectra recorded with the vesicle fractions are identical to those reported for LY [1].

Fluorescence Measurements. To define further the photophysical properties of the LY-labeled vesicles, time-resolved fluorescence measurements were carried out. The results obtained are summarized in Table I. In the table the data obtained with free LY-CH and with LY-iodoacetamide reacted with β-mercaptoethanol (LY-βME), used as a model compound, are also shown for comparison. The fluorescence decay of LY-CH and LY-βME is strictly monoexponential, with fluorescence lifetimes of 9.5 and 6.4 ns, respectively. On the contrary, a poor fitting is obtained when the fluorescence decay of LY covalently bound to the lipid vesicles is analyzed as a single-exponential function ($\chi^2 = 8.86$). In this case, in fact, the fluorescence intensity decay is best fitted by a biexponential decay with lifetimes of 7.9 and 1.1 ns ($\chi^2 = 1.18$). In Fig. 3, the results of the analysis of the fluorescence decay curves obtained with LY-βME (Fig. 3A) and with DMPE-LY/DMPC vesicles (Fig. 3B) to a single-exponential model are shown for comparison.

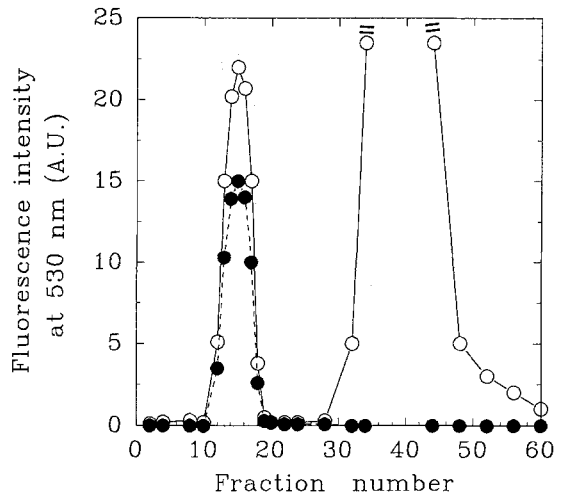

Fig. 1. Column chromatography of the LY-labeled liposomes. Vesicles (1.5 ml) were applied to a 1.5 × 25-cm column of G-50 Sephadex run at 1.82 ml min^{-1}, at 4°C. Small aliquots (100 μl) of each fraction (0.8 ml) were added to 1.9 ml of buffer. The open circles represent the fluoresence intensity recorded exciting the samples at 430 nm and observing the emission at 530 nm. The filled circles represent the phorphorus content of each fraction as revealed on TLC silica plates by the phosphorus staining reaction and they do not have a quantitative value.

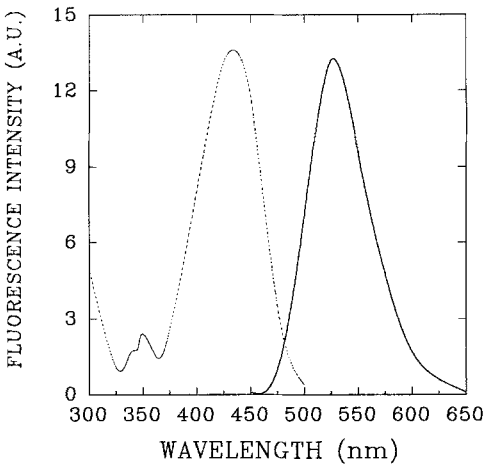

Fig. 2. Excitation (dashed line) and emission (solid line) spectra of LY labeled liposomes. The vesicle sample was prepared mixing 200 μl of fraction 12 (see Fig. 1) with 1.8 ml of buffer. Spectra were recorded using 5-nm bandwidths on both the excitation and the emission path.

From the time-resolved data, an intensity-weighted mean lifetime, τ_m of 7.15 ns was calculated for DMPE-LY/DMPC as, $\Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$ [18], and a relative QY of 0.16 was obtained, assuming no changes of the natural lifetime of LY. The steady-state emission anisotropies measured with LY-labeled vesicles and with the free LY dyes, LY-CH and LY-βME, are also shown in Table I. The low values observed with LY-CH and LY-βME reflect the rotational freedom of small molecules dissolved in solution, whereas the higher $<r>$ observed with DMPE-LY/DMPC liposomes is consistent with restricted rotational movements due to the labeling with vesicles.

Quenching measurements have often been used to discriminate between accessible and unaccessible fluorophores. In particular, it has been pointed out how a linear Stern–Volmer plot reflects the existence of fully accessible emitting species, whereas a downward curvature of the plot reflects the existence of unaccessible

Table I. Time-Resolved Intensity and Steady-State Anisotropy Fluorescence Parameters[a]

	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2	<r>
LY-CH	1.00	9.49	—	—	1.22	0.020
LY-βME	1.00	6.43	—	—	1.23	0.005
DMPE-LY/DMPC	0.52	7.93	0.48	1.12	1.18	0.129
DMPE-LY/DMPC	1.00	6.78	—	—	8.86	0.129

[a] For τ_i and α_i are indicated the fluorescence lifetimes and the associated preexponential terms, respectively, where $\Sigma\alpha_i = 1$. For DMPE-LY/DMPC are indicated the data relative to the LY-labeled vesicles (SUV). All the experiments were performed in 10 m*M* Tris–HCl, pH 7.4, at 24°C. Search of the minimal value of χ^2 was used to judge the goodness of the decay data fits [17]. The steady-state anisotropy, <r>, was measured exciting the samples at 430 nm and observing the emitted fluorescence at 530 nm; bandwidths of 10 nm each were used in the excitation and the emission paths, respectively.

emitting species [16]. In the present work we have used KI, a polar negatively charged molecule which does not diffuse inside the lipid membrane, as a quencher. In Fig. 4, the Stern–Volmer plots relative to the quenching of SUV and LUV LY-labeled vesicles are shown. In the concentration interval between 0 and 0.44 *M* KI both the vesicle preparations gave rise to linear Stern–Volmer plots with intercept values very close to one. From these data, Stern–Volmer (K_{sv}) and bimolecular rate (k_q) quenching constants were obtained, and are presented in Table II. In Table II the quenching constants were obtained with LY-βME free in solution in the KI concentration range between 0 and 0.135 *M* (data not shown) are reported for comparison. It is of interest to note that the k_q obtained with the LUV is higher than the k_q obtained with the SUV liposomes. This results can be explained by the fact that larger the vesicle surface, the higher becomes the probability of encounter between the quencher and the fluorophore molecules. In addition, the bimolecular quenching rate constant obtained with LUV is comparable to the rate constant obtained with free LY-βME (0.31×10^{10} vs $0.43 \times 10^{10}\ M^{-1}\ s^{-1}$) and close to that expected for a diffusion-controlled quenching process [19]. This result suggests that the LY chromophore is fully exposed to the solvent.

Cell Labeling. The results obtained using the labeled vesicles as a carriers of LY to stain cell membranes are presented. In order to distinguish cell labeling due to actual LY-lipid transfer from labeling due to vesicles adhering to the cell surface, staining experiments were carried out exclusively using LUV. Under these conditions fluorescent liposomes (diameter, ≈200–400nm [14]) are evident under the optical microscope (data not shown), thus revealing possible changes of the labeled cell's profile. We separately evaluated sperm cells and lymphocytes. Upon incubation with LY-labeled liposomes, the cells become fluorescent. In Fig. 5, the result of a typical incubation experiment with spermatozoa is shown. The cells and the vesicles have been incubated for increasing times at 4 and 36°C. A noticeable decrease in fluorescence staining was observed when the incubation is performed at 4°C. At 36°C, the fluorescence appears after 10 min and reaches its maximum intensity after 1 h of incubation. A marked LY fluorescence is evident in the *head* region and in the mitochondrion-containing *midpiece* portion of the spermatozoan tails. In the evaluated samples, an averaged labeling yield of about 35% was observed. Cell viability, as judged by the trypan blue dye exclusion assay, ranged between 60 and 80%, and the integrity of the acrosomal membrane, after incubation with the vesicles, was found to be 89% of the sperm cells. Furthermore, when free LY was mixed with the spermatozoa, no fluorescence labeling was obtained. We did not find any significant difference of the labeling characteristics, both when liposomes of different phospholipid compositions (i.e., DMPE-LY/DMPC/DMPS) were used and when $CaCl_2$ was added to the incubation sample. In Figs. 6 A–D, sperm cells incubated with fluorescent vesicles are shown at a higher magnification. In Fig. 6B, the arrow indicates a shadowed, *cap-like*, fluorescence around the intense head profile. It would be tempting to ascribe this pale fluorescence to the acrosomal membranous structure.

In Figs. 7A to D, the images obtained with lymphocytes incubated with fluorescent liposomes are presented. After 1 h of incubation at 36°C the cells become

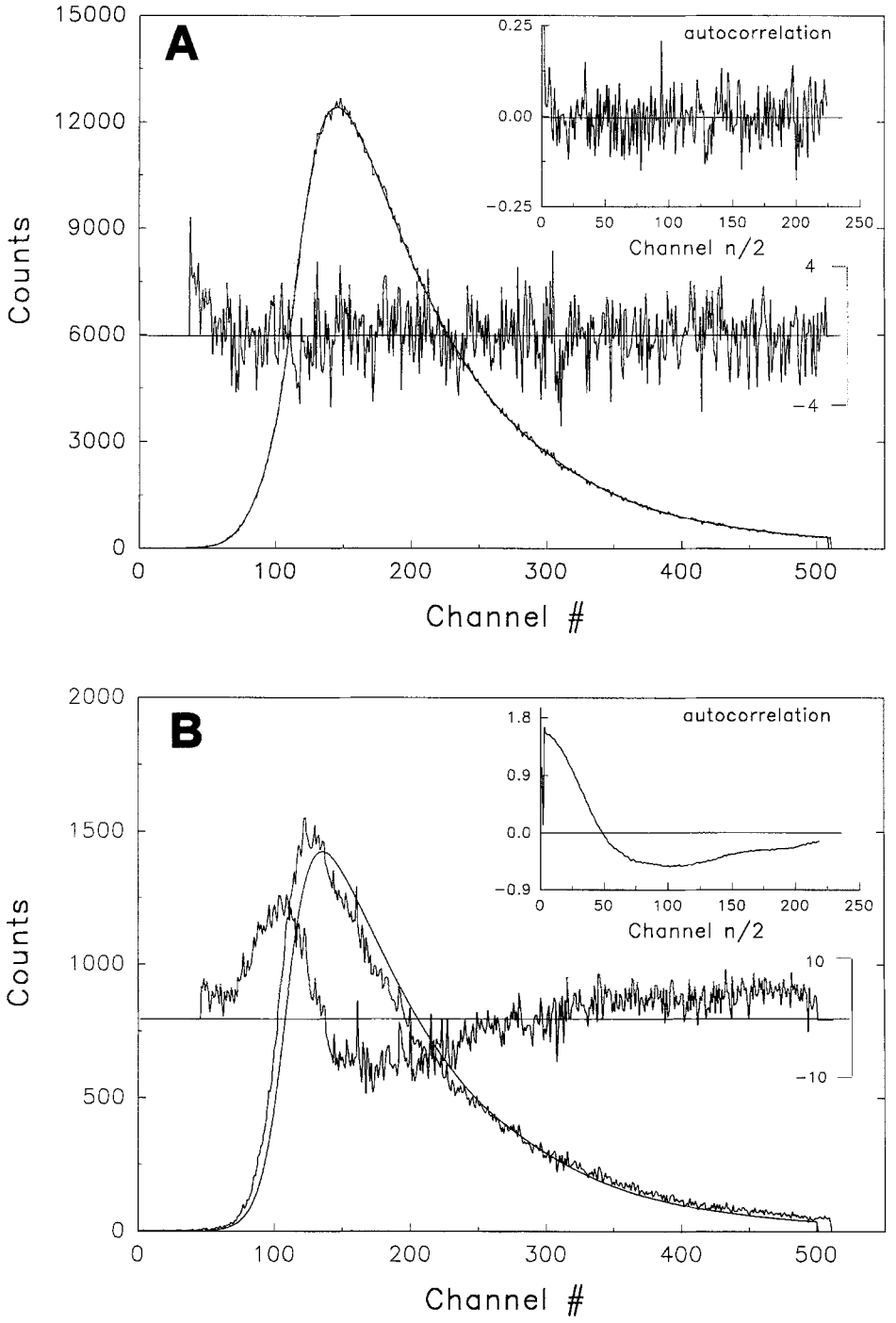

Fig. 3. Analysis of the fluorescence intensity decay of LY-βME and DMPE-LY/DMPC vesicles (SUV). Fluorescence decay data were obtained in 10 m*M* Tris–HCl, pH 7.4, at 24°C, using an excitation wavelength of 430 nm (bandpass, 4 nm), and fluorescence emission was observed at 530 nm (bandpass, 10 nm). The calibration time of each channel was 0.0862 ns. Photon counts are reported on a linear scale. The solid noise-free curves represent the theoretical parameters convolved with the excitation impulse function; the experimental decay data are represented by the noisy curves. The results of the analysis of the data to a monoexponential model are presented for LY-βME (A) and for DMPE-LY/DMPC (B). The resulting decay parameters were as follows: LY-βME, τ_1 = 6.43; and DMPE-LY/DMPC, τ_1 = 6.78. The plots of the weighted residuals (center graphs) and of the autocorrelation function of the weighted residuals (inset), used to judge the goodness of the fits, are also shown.

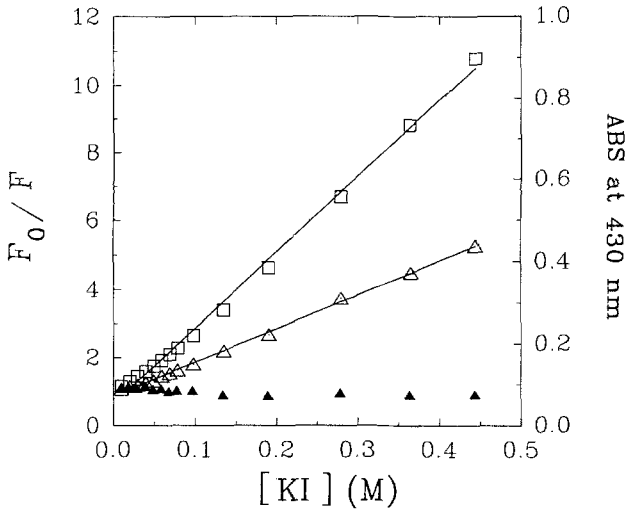

Fig. 4. Stern–Volmer plots of the LY-labeled liposomes by Kl. Samples at increasing Kl concentrations were prepared by the addition of small volumes of the quencher (5 μl) to a 6.3×10^{-4} *M* vesicle suspension. The open squares represent the data obtained with LUV, and the open triangles the data obtained with SUV. The filled triangles represent the absorbances measured at each Kl concentration with SUV. The straight lines represent the best results of a linear regression analysis using Eq. (1).

fluorescent, with an apparent labeling yield of 15%. In Fig. 7B, the image of a labeled lymphocyte is presented. To test if different conditions could improve the labeling yield, lymphocytes immobilized on polylysine-coated culture dishes were subjected to incubation (60 min) with liposomes. In Figs. 7C and D the result obtained under this condition is shown. The image was taken directly through the plastic dish, thus filtering part of the fluorescence intensity; nonetheless, an apparent decrease in the fluorescence incorporated per cell is evident. Concomitantly, the number of cells labeled is increased.

All the images shown were taken immediately after incubation and a few minutes of continuous illumination; nevertheless, negligible fluorescence photobleaching was observed and the samples are clearly visible under the microscope for a long time. Spermatozoa cells were irradiated for 1–2 h without any apparent fading of the fluorescence intensity.

DISCUSSION

LY, as a dye for cell biology studies, shows highly desirable photophysical features, such as a large Stokes shift and a high quantum yield. In addition, the peculiar high polarity of the molecule provided an ideal tool to study the aqueous intracellular compartment by fluorescence techniques [2]. The latter characteristic, however, has limited the use of the dye as a tracer of the cytosol, and staining of the cell surface by LY has been achieved only by reacting the dye with enzimatically or chemically oxidized membrane glycoconjugates [8].

Thus, the aim of this work was the preparation of LY fluorescent liposomes containing DMPE-LY derivative and the vehiculation of the labeled phospholipid to cell membranes using the vesicles as a carrier.

We took advantage of the original method described by Carlson *et al.* [12] for the thiolation of the NH_2 of proteins and used it to introduce SH-reactive groups into DMPE-containing vesicles. Following this procedure a wide variety of compounds and macromolecules which readily react with reduced SH groups can be linked to liposomes [13].

We have shown that, once labeled to the lipid ves-

Table II. Fluorescence Intensity Quenching Constants[a]

	K_{SV} (M^{-1})	k_q ($\times 10^{-9}$ M^{-1} s^{-1})
LY-βME	27.50	4.28
DMPE-LY/DMPC[b]	9.90	1.38
DMPE-LY/DMPC[c]	22.24	3.11

[a] The Stern–Volmer constant, K_{SV}, was obtained from the linear regression of the data presented in Fig. 3, as the slope of each line [see Eq. (1)]. The bimolecular quenching rate, k_q, was calculated as $k_q = K_{SV}/\tau_0$, where τ_0 is the fluorescence lifetime measured in the absence of the quencher.

[b] SUV, LY-labeled vesicles.

[c] LUV, LY-labeled vesicles. In these cases, τ_m, calculated from decay curves measured in the absence of the quencher, was used as τ_0.

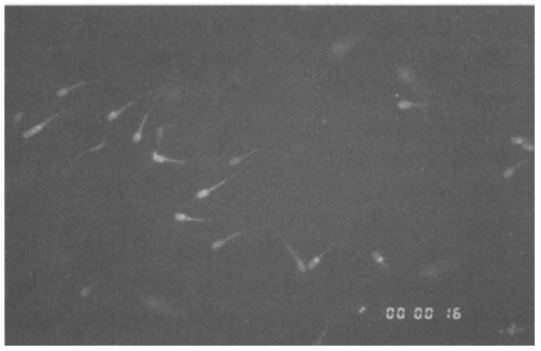

Fig. 5. Fluorescence microphotograph of sperm cells incubated for 60 min, at 36°C, with LY-liposomes (3–4 m*M*). After incubation, the cells were washed twice with 10 m*M* Tris–HCl, pH 7.4, 0.137 *M* NaCl. 80×; reduced 35% for reproduction.

icles, the dye retains the basic steady-state excitation and emission characteristics of the free LY dyes. In addition, the nanosecond time-resolved fluorescence data show that simple chemical derivatization (formation of the thioaliphatic bond) does not modify the single-exponential decay kinetics of the fluorescence intensity of LY. This information was obtained using the model compound LY-βME (Table I). On the other hand, the decay kinetics becomes biexponential when the LY fluorescence transient is measured with the fluorophore embedded in the vesicles (Table I).

Since time-resolved experiments were recorded under "magic-angle" conditions [17], the effect of rotational anisotropy on decay curves can be reasonably excluded.

Biexponential decay has often been reported for fluorescent membrane probes [20–23]. These results have been referred to the detection of different coexisting phases (lipid domains), and several excellent discussions on the origins of complex photophysical behaviors in membranes have been presented [22–24]. In addition, changes of the fluorescence lifetimes have been successfully associated with distinct lipid phases [25]. Indeed, this evidence has been provided for probes which are located mainly in the acyl core of the bilayer, thus in a region that is ideal for monitoring the lipid's structural fluctuations. Nonetheless, changes of the physical state of the bilayer have been reported to affect also the fluorescence properties of lipid derivatives with the dye attached at the polar head region, and specifically designed to study biological processes at the lipid–water interface of membranes [26; unpublished observation]. Further support for these observations came recently from molecular dynamics studies [27] which showed how, on going from

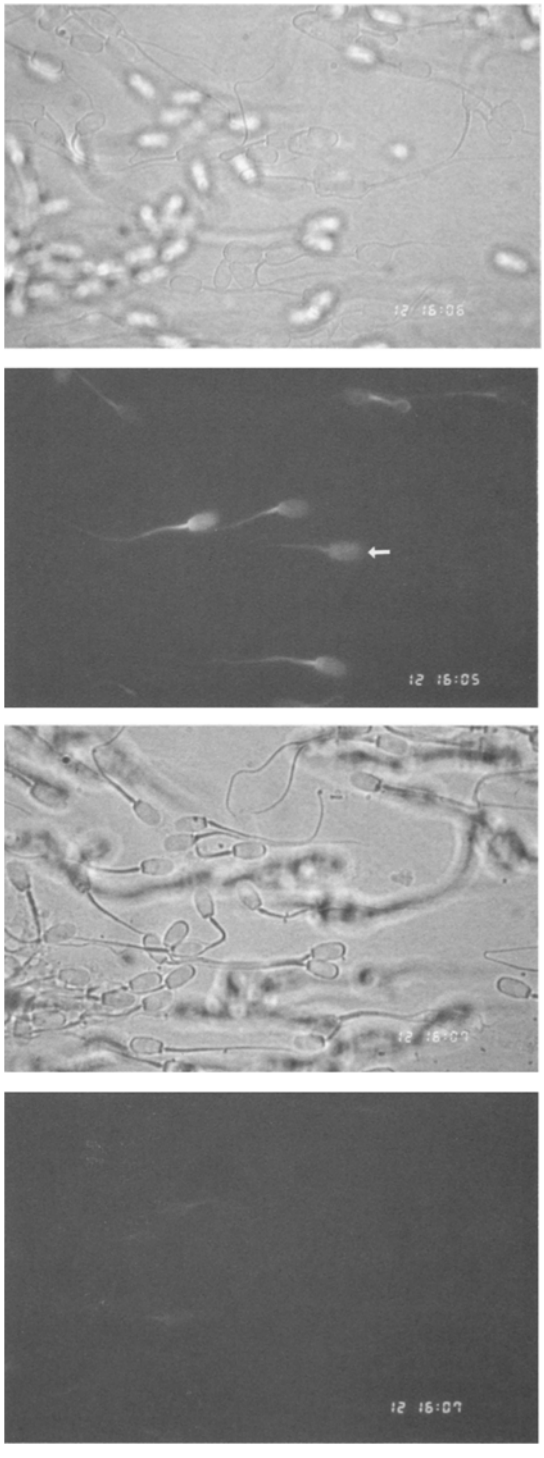

Fig. 6. Phase-contrast (A and C) and fluorescence (B and D) photomicrographs of spermatozoa cells. Two focal planes are shown; see text for details 200×; reduced 35% for reproduction.

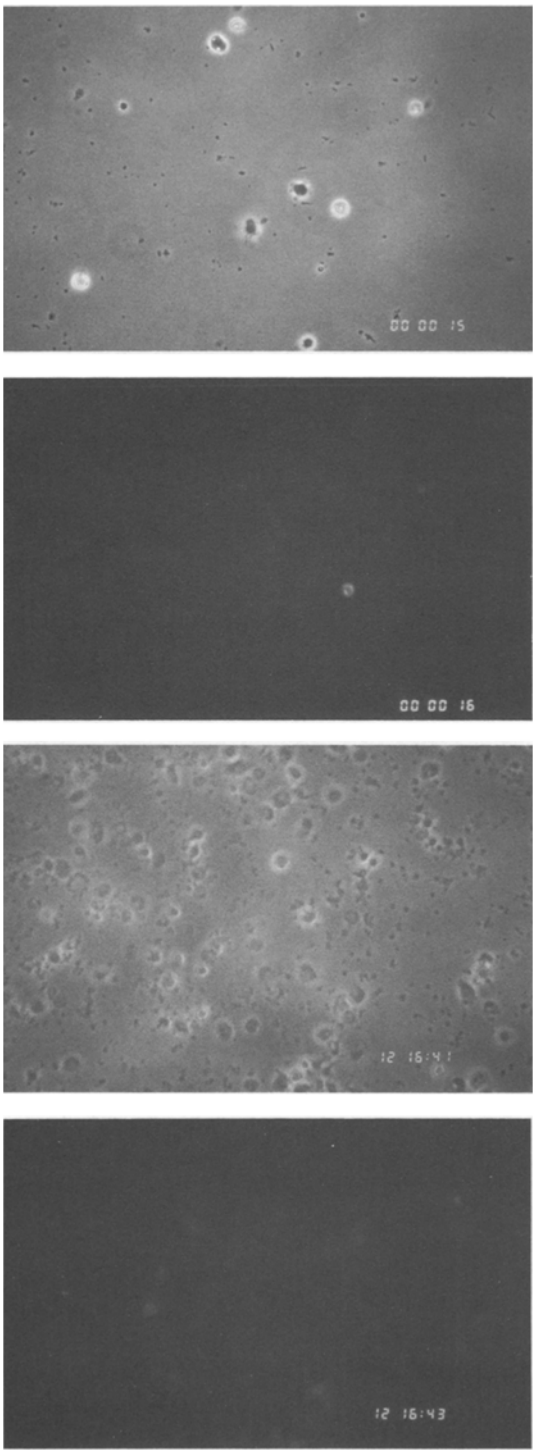

Fig. 7. Phase-contrast (A and C) and fluorescence (B and D) photomicrographs of lymphocytes incubated with fluorescent liposomes under different conditions: A and B, lymphocyte suspension in PBS (1×10^6 cells/ml); C and D, lymphocytes immobilized on polylysine coated culure dishes; 200×; reduced 35% for reproduction.

the highly ordered L_β phase to the partially ordered liquid crystal L_α phase, both the segmental acyl chain and the whole molecule (axial rotation and wobble) reorientational movements are affected. For these reasons, it would be tempting to related the biexponential decay behavior of DMPE-LY in liposomes to two coexisting lipid phases. Based on our experimental results, we restrict the discussion to processes which may end in the quenching of DMPE-LY species. Probe-enriched regions could be characterized by fast-decaying fluorescence emission due to different collisional interactions. These may include charge repulsions between the SO_3^- groups of LY. According to this representation, closely spaced DMPE-LY species may be responsible for the appearance of the short decay component ($\tau_2 \approx 1$ ns), while well-spaced DMPE-LY species may be responsible for the long decay component ($\tau_1 \approx 8$ ns), which is very close to the value of the single lifetime observed in aqueous solution.

In addition to the "lipid domain" model, the evidence that the phospholipid polar groups can align parallel to the plane of the membrane [28] may represent an alternative explanation for the biexponential decay observed with LY-labeled liposomes. In fact, in could be related to the existence of two DMPE-LY species which, depending on the different interaction with the membrane surface, may experience distinct excited-state depletion kinetics. The short lifetime may be associated with "highly interacting" species characterized by the LY polar group aligned closely to the membrane plane, and the long lifetime may be associated with "poorly interacting" species characterized by the LY polar group freely exposed to the solvent. In this case the short decay component may originate from a collisional mechanism driven by the repulsion between the negatively charged LY polar group, kept in the bent orientation by structural constraints, and the hydrophobic membrane surface.

At present, a unique explanation cannot be provided and extensive scrutiny of the fluorescence decay of LY-labeled vesicles under various experimental conditions ($T°$, liposomes of different lipid composition, etc.) will be required to understand this complex decay kinetics better.

The quenching data we have reported add significant information on the location of the LY-labeled DMPE molecules. Using KI as a quencher, high bimolecular quenching constants were obtained. This result, together with the linear Stern–Volmer plots recovered, implies that the fluorophore is fully accessible to the I^- ions (i.e., the LY dies are all located at the external surface of the bilayer).

Nonetheless, concomitant processes might take place

which could affect LY distribution on liposomes, and a comment is worthwhile to rule out these possibilities. Lipids can be internalized by flip-flop movements. This process has been reported to occur in a wide range of times (from a few to several hours) [29]. In the work described the quenching assays were carried out within 12 to 24 h from the labeled vesicle preparation. In the case of flip-flop transverse diffusion, a number of DMPE-LY molecules might be sequestrated inside the liposomes and, thus, excluded from collisions with the quencher. This condition would generate unquenchable species and downward-curved Stern–Volmer plots should be expected [16]. On the contrary, linear Stern–Volmer plots were always obtained under the different experimental conditions evaluated. Moreover, the high polarity of the LY moiety makes transverse motions of DMPE-LY molecules extremely unlike.

Similarly, the heavily hydrated and negatively charged iodide ions should be able to quench only surface residues. In addition, it has been shown that I^- penetrates lipid bilayers slowly if at all [30]. According to our experimental plans small aliquots of the quencher were added to the sample and the fluorescence intensity was instantly recorded. Thus, this procedure should exclude the effects of slow diffusion processes. Nevertheless, for the sake of discussion, we might assume that some penetration of the iodide ions through the hydrophobic barrier of the bilayer would occur. In this case, two boundary conditions may be verified: (i) KI diffusion occurs in the prescence of vesicles with the LY dye located exclusively at the external surface of the membrane; and (ii) KI diffusion occurs in the presence of vesicles with the LY dye located at both the external and the internal surface of the membrane (less favorable condition; see the discussion above). In the first case, KI diffusion should subtract quencher molecules from the external solvent altering the actual KI concentration. Under this condition the Stern–Volmer plots should be downward-curved or, at least, become biphasic. As shown under Results, neither of these features was verified. According to the second hypothesis, no deviation from Stern–Volmer linearity should not be obtained only in the case of extremely fast diffusion or complete liposome disruption. The latter occurrence was certainly excluded for the experiments with labeled LUV by fluorescence microscopy inspection, and it is very unlikely also for the experiments with labeled SUV. Alternatively, in the case of slower diffusion of the quencher inside integer liposomes, the quenching parameters should become time dependent. At an early stage of the experiment condition i should be reproduced, and at a later stage of the experiment a rising of the quenching parameters should be observed when I^- molecules reach the inner side of the vesicles.

The results we have presented do not contain any of these contributions and thus they seem to exclude both the existence of significant concentrations of "internal" and "external" emitting species and the presence of significant diffusional processes of KI inside the liposomes.

However, in view of the fact that time-resolved measurements have shown that 90% of the fluorescence intensity is due to the contribution of the long decay component (Table I), purly graphycal evaluation of the Stern–Volmer plots may not pick up minor deviations from linearity. In the future it will be extremely useful to carry out time-resolved quenching experiments to unravel the existence of complex mechanisms.

In addition, it is worth noticing that the defined external location of the probe may also provide an ideal tool for studying the interaction of soluble macromolecules (i.e., proteins, hormones etc.) with membrane models by fluorescence techniques (i.e., fluorescence resonance energy transfer).

The DMPE-LY-bearing vesicles have been used as a carrier of the fluorescent phospholipid to cell membranes. We have shown that within 1 h, at 36°C, spermatozoa become fluorescent. At lower temperatures, longer incubation times are required for cell staining. This observation can be related to the higher membrane viscosity and its effect on lipid transfer. Interestingly, the *midpiece* tail region of the sperm cells is characterized by brighter fluorescence (Fig. 5), suggesting the specific incorporation of DMPE-LY in regions with a high lipid concentration (i.e., mitochondrion-containing compartments).

We report that the highest extent of sperm labeling was obtained with apparently integer and viable cells, characterized by a low motility. Since no staining is observed by incubation of spermatozoa with the free fluorescent dye, we exclude that the labeling is due to aspecific permeation of the dye into viable, but damaged, cells. On the other hand, if we assume that staining is proportional to the extent of cell–liposome interaction, it seems reasonable that cells with a low motility, which experience a longer contact with the vesicles, are better labeled.

The temperature-dependence effect on staining was also observed with lymphocytes. In spite of the poorer labeling yield with respect to spermatozoa, lymphocytes also show some degree of labeling with the LY fluorescence. An apparent increase in the labeling yield was observed with lymphocytes immobilized on polylysine-coated culture dishes (Fig. 7). In fact, under this con-

dition a larger number of cells is labeled with an apparent decrease in the LY fluorescence incorporated per cell.

We report that after labeling, light irradiation caused a negligible photobleaching of the object under the microscope. This result may represent a significant accomplishment for fluorescence microscopy observations. To our knowledge, this effect has not been reported previously. All the fluorescence microscopy measurements presented in this work were carried out using a broad-bandpass fluorescein filter [31]. Such a configuration, while not providing the most efficient excitation of LY, could determine a weaker irradiation of the biological sample, which results in very low photodamage. Nonetheless, when the appropriate LY filter became available in the course of this work, only a slight increase in photobleaching was observed. In addition, quenching experiments of LY dyes by acrylamide (our unpublished observation) surprisingly revealed a total reluctance of the fluorophore to the quenching. Thus, it is possible that the negligible photobleaching observed is due to intrinsic features of the molecule.

As a final remark, we would like to point out that the procedure reported could be considered as a general method to react the polar-head group of lipid with compounds that are not soluble in organic solvents.

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REFERENCES

1. W. W. Stewart (1978) *Cell* **14,** 741–759.
2. W. W. Stewart (1981) *Nature* **292,** 17–21.
3. J. H. Peacock, D. F. Rush, and L. H. Mathers (1979) *Brain Res.* **169,** 231–246.
4. A. C. Crawford and R. Fettiplace (1980) *J. Physiol. Lond.* **306,** 79–125.
5. A. D. Murphy and S. B. Kater (1980) *Brain Res.* **186,** 251–272.
6. A. G. M. Bulloch and S. B. Kater (1981) *Science* **212,**79–81.
7. J. A. Swanson, B. D. Yirinac, and S. C. Silverstein (1985) *J. Cell Biol.* **100,** 851–859.
8. S. Spiegel, M. Wilchek, and P. H. Fishman (1983) *Biochem. Biophys. Res. Commun.* **112,** 872–877.
9. J. C. Dittmer and R. L. Lester (1964) *J. Lipid Res.* **5,** 126–127.
10. C. H. Lea and D. N. Rhodes (1955) *Biochim. Biophys. Acta* **17,** 416–423.
11. C. W. F. McClare (1971) *Anal. Biochem.* **39,** 527–532.
12. J. Carlsson, D. Hakan, and R. Axen (1978) *Biochem. J.* **173,** 723–737.
13. L. D. Leserman, J. Barbet, and F. Kourilsky (1980) *Nature* **288,**602–604.
14. L. T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds (1981) *Biochemistry* **20,**833–840.
15. B. A. Didion, J. R. Dobrinsky, J. R. Giles, and C. N. Graves (1989) *Gamete Res.* **22,**51–57.
16. S. S. Lehrer (1971) *Biochemistry* **10,**3254–3263.
17. M. G. Badea and L. Brand (1979) *Methods Enzymol.* **75,**378–425.
18. M. Inokuti and F. Hirayama (1965) *J. Chem. Phys.* **43.** 1978–1989.
19. J. R. Lakowicz (1983) *Principles of Fluorescence Spectroscopy,* Plenum Press, New York and London.
20. L. A. Chen, R. E. Dale, S. Roth, and L. Brand (1977) *J. Biol. Chem.* **252,** 2163–2169.
21. L. A. Sklar, B. S. Hudson, and R. D. Simoni (1977) *Biochemistry* **16,**819–828.
22. R. D. Klausner, A. M. Kleinfeld, R. L. Hoover, and M. J. Karnovsky (1980) *J. Biol. Chem.* **255,** 1286–1295.
23. T. Parasassi, F. Conti, M. Glaser, and E. Gratton (1984) *J. Biol. Chem.* **259,** 14011–14017.
24. R. A. Parente and B. R. Lentz (1985) *Biochemistry* **24,** 6178–6185.
25. A. Ruggiero and B. Hudson (1989) *Biophys. J.* **55,** 1111–1124.
26. P. Neyroz, L. Franzoni, A. Spisni, L. Masotti, and L. Brand (1992) *Chem. Phys. Lipids* **61,** 255–263.
27. R. D. Pastor, R. M. Venable, and M. Karplus (1991) *Proc. Natl. Acad. Sci. USA* **88,** 892–896.
28. J. Seelig, P. M. Macdonald, and P. M. Scherer (1987) *Biochemistry* **26,** 7535–7541.
29. P. F. Devaux (1991) *Biochemistry* **30,** 1163–1173.
30. G. L. Jendrasiak (1972) *Chem. Phys. Lipids* **9,** 222–236.
31. J. Swanson (1989) in Yu-li Wang and D. L. Taylor (Eds.) *Fluorescence Microscopy of Living Cells in Culture,Part A, Methods in Cell Biology, Vol. 29,* Academic Press, San Diego, CA, pp. 137–150.