Probing the structure of diacetylenic phospholipid tubules with fluorescent lipophiles

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ABSTRACT Novel lipid structures called tubules can be prepared from diacetylenic phospholipids. We have prepared fluorescent tubules from mixtures of 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphatidylcholine and 1 mol% fluorescent lipophiles to study the characteristics of the tubule lipid matrix. We have found that once formed, tubules do not incorporate lipophiles from the aqueous phase into their lipid matrix. The spectral characteristics of the fluorophore laurodan in tubules, and the lack of diffusion of *N*-nitrobenzoxydiazol phosphatidylethanolamine in tubules, have allowed us to characterize the microenvironment of these structures as being extremely rigid and tightly packed. Despite their rigid characteristic, tubules are formed from intact liposomes as demonstrated by the formation of doubly labeled tubules from two populations of liposomes, each of which contained a different nonexchangeable fluorescent lipophile.

INTRODUCTION

A novel lipid structure which forms from diacetylenic phospholipids has been reported (1, 2). The structure has been characterized as a "tubule" because it is long and thin (~1 × 100 μm in length) and appears to be hollow by light and electron microscope observations. It has been suggested that tubules form from intact bilayers which flatten and roll up (2) to produce a structure which is cylindrical and open on the ends. These structures are clearly distinguished from cochleate cylinders (2).

Lipid tubules may have future application in electroptics, microelectronics, reagent delivery, or microsurgery (3). For many potential applications the ability to incorporate desired proteins and lipids into the matrix would be extremely advantageous. It is important to characterize the molecular environment of this unique lipid matrix with respect to its permeability, its tendency to incorporate materials from its surrounding medium, and its suitability as a matrix for active membrane proteins. Using a variety of fluorescence techniques, we have studied some aspects of the physical characteristics of tubules to better understand the microscopic environment of the tubule matrix, and to examine the mechanism of their formation.

MATERIALS AND METHODS

The following materials were purchased from Molecular Probes (Junction City, OR): 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI[3]), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiI[5]), 1-octylpyrene, 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan), Nile Red, and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBDPE). Pyrene came from Aldrich Chemicals (Milwaukee, WI). Distearoyl phosphatidylcholine (DSPC) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Birmingham, AL). The polymerizable lipid, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphatidylcholine ($DC_{23}PC$) was synthesized at the Naval Research Laboratory by Dr. Alok Singh as previously described (4).

To prepare tubules, chloroform solutions of 2.5 mg of $DC_{23}PC$ were taken to dryness in conical vials under N_2 and desiccated overnight under vacuum. 1 ml of deionized water was added to the vials, and the vials were placed at ~60°C for 2 h. After that time, the temperature was decreased slowly to 37°C. This method of preparation of $DC_{23}PC$ tubules has been described previously (2). Tubule formation begins to occur during the cooling process and usually almost all of the lipid is present as tubules after 1 h at 37°C. The formation of tubules is dependent on the presence of very large liposomes, and is sensitive to mixing and to temperature regime. Vortexing of the $DC_{23}PC$ lipid suspension, for example, will prevent the formation of tubules (2). Under optimum conditions, which includes very precise temperature control, Burke et al. (5) have reported quantitative conversion of $DC_{23}PC$ liposomes to tubules. Our tubules were prepared without extremely precise temperature control, and therefore a small amount of lipid, probably <10% of the total, is present in the preparation as liposomes. No attempt was made to quantify this amount. Tubules containing fluorescent lipophiles were prepared by adding fluorophore to $DC_{23}PC$ lipid solutions in chloroform at a final concentration of 1 mol% of total lipid. Tubules were then prepared from this lipid mixture in the same manner as those from $DC_{23}PC$ lipid only. A small amount of unconverted liposomes were present in these tubule preparations also, but no more than was seen in preparations of tubules not containing fluorophores. The tubules were stable for weeks at temperatures below their phase transition of 57°C.

Large multilammelar liposomes were prepared from $DC_{23}PC$ and DSPC by drying lipid from chloroform and desiccating dried samples overnight under vacuum. Lipid was rehydrated at ~50°C, and swirled or

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mixed by vortex. Because the $DC_{23}PC$ suspension was well mixed with water and was not subjected to careful cooling, tubules did not form. This was confirmed by light microscopy. Light scattering and photon correlation spectroscopy was performed as described by Chang et al. (6), and indicated that these liposomes were very heterogeneous in size and contained many large (~1 μm diam) structures. Smaller lipid vesicles were prepared from POPC by the injection method (7).

Fluorescence spectroscopy was performed at 20°C with a Spex Industries, Inc. (Edison, NJ) Fluorolog II. Fluorescence microscopy experiments which involved digital image analysis were performed on a Leitz, Inc. Diavert (Rockleigh, NJ) using a 63×/1.4 numerical aperture objective lens. Aqueous suspensions of tubules or liposomes were placed on a no. 1.5 coverslip in a Bionique chamber (Lake Placid, NY), followed by another coverslip. The excitation source was a 100-W Mercury arc. All images were collected at ambient temperature (~25°C), except for Fig. 5. The temperature changes required for the

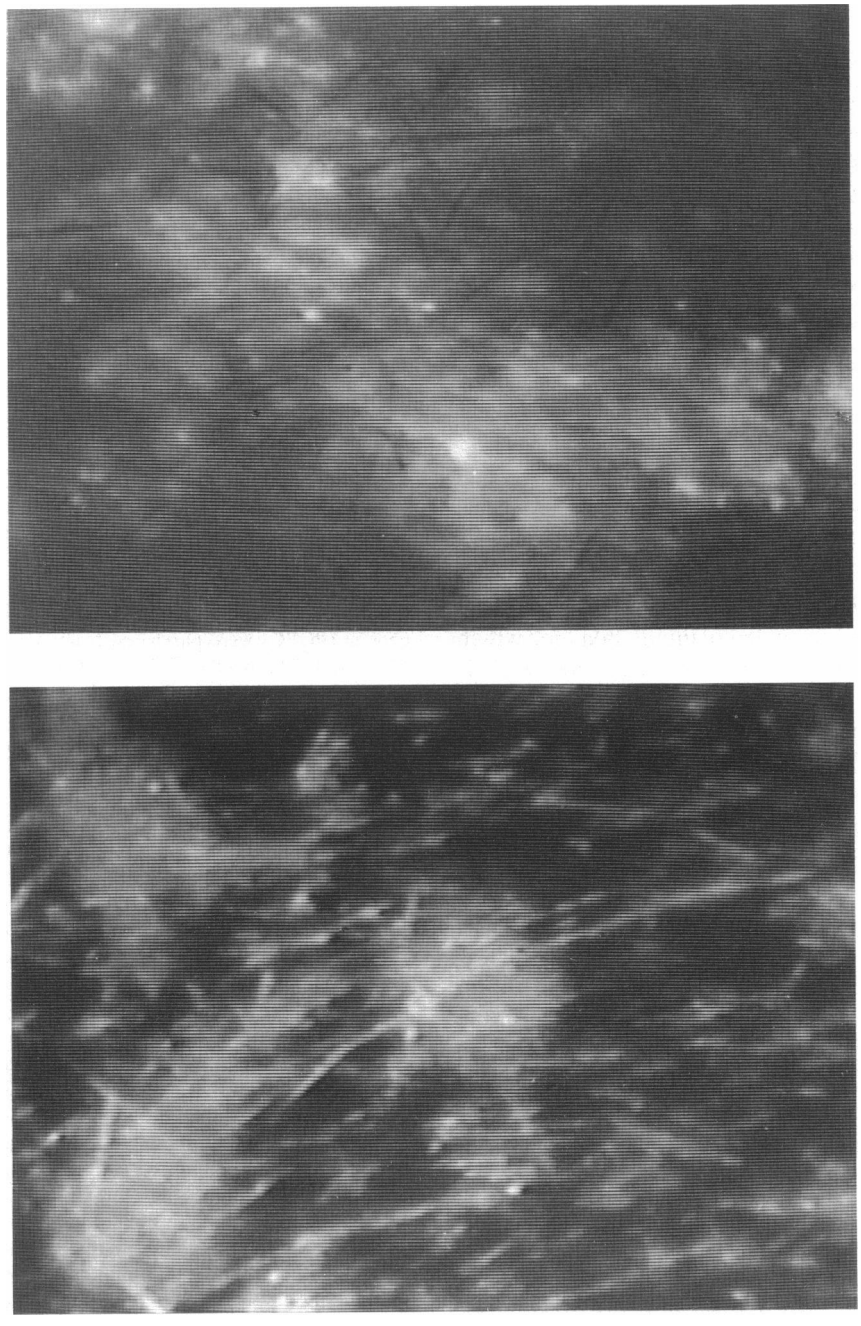

FIGURE 1 Tubule preparations were viewed at a magnification of 630×. A filter cube combined a 450–490-nm bandpass filter and a 515-nm cut-on filter. Photographs of nondigitized images were obtained from a video monitor. (*Top*) Tubules formed from $DC_{23}PC$ were incubated with an aqueous solution of Nile Red (0.14 mg/ml). (*Bottom*) Tubules were formed from a mixture of Nile Red and $DC_{23}PC$.

experiment shown in Fig. 5 were achieved with heat guns aimed at the stage, and the temperature was measured with a small probe taped to the stage near the sample. Image analysis was performed on video images collected with a Vidicon camera (model c 1000-IZ; Hamamatsu Corp., Middlesex, NJ) and digitized by averaging 32 frames with a 274 image processor (Grinnel Systems Corp., San Jose, CA). Photographs of the Grinnel images were produced with a Matrix Instruments (Mesa, AZ) 35 mm color graphic recorder. For a complete description of the data acquisition system, see reference 8. The fluorescence micrographs in Fig. 1 were obtained with a camera (model SIT 66; Dage-MTI, Inc., Michigan City, IN) on a similar microscope. Video images were recorded on a video recorder (model NV 8950; Panasonic Co., Secaucus, NJ), and the photographs were taken directly from a monitor (model PVM-122; Sony Corp., Long Island City, NY). Excitation and emission bandpasses were selected with appropriate filters as indicated in the figure legend. Data analysis was performed on an Inovision (Research Triangle Park, NC) IC-1 image workstation.

RESULTS

We have successfully incorporated many structurally dissimilar fluorescent lipophiles into the lipid matrix of $DC_{23}PC$ tubules. DiI(3), DiI(5), pyrene, octylpyrene, pyrene-DPPE, laurdan, Nile Red, and NBDPE have all been added at a level of 1 mol% to $DC_{23}PC$ lipid in solvent and have been trapped in the lipid matrix during tubule formation. In all cases (except for pyrene due to technical difficulties), fluorescent tubules were observed by microscopy, which indicated that the fluorophores had been incorporated into the lipid matrix of the tubules. The fluorescence spectra of these probes in tubules were identical to their spectra in organic solvents (data not shown), suggesting that the probes were in a nonpolar environment.

One characteristic of lipid bilayer membranes and other lipid structures such as micelles and lipoproteins is their tendency to accept lipophilic materials from the aqueous phase. Lipophiles such as fatty acids, phospholipids, and cholesterol have been shown to transfer spontaneously through the aqueous phase into hydrophobic matrices such as membranes and lipoproteins (9), and thus it is reasonable to assume that such molecules might spontaneously associate with tubules. The association of the lipophile, Nile Red, with lipid matrices such as cell membranes and intracellular lipid droplets has been reported (10). Nile Red is sparingly soluble in H_2O, and is only weakly fluorescent until it transfers into a hydrophobic environment. Nile Red in aqueous solution at 12 μM transferred at room temperature into $DC_{23}PC$ liposomes (87 μM lipid) with a 37-fold increase in fluorescence intensity over 15 h. However, when the same amount of $DC_{23}PC$ lipid in the form of tubules was added to the same concentration of aqueous Nile Red, only a seven-fold increase in intensity was observed in 15 h. As shown in the top panel of Fig. 1, fluorescence microscopy demonstrated that the increase in intensity was due to Nile Red which transferred into nontubule lipid material in the sample. For this image, the tungsten lamp provided enough illumination to allow observation of the phase and fluorescence simultaneously. The faint straight structures are tubules and the fluorescence is associated entirely with amorphous liposomal material. Even after incubation for 1 mo at room temperature, no Nile Red fluorescence was seen in tubules. Tubules containing Nile Red could be produced (as shown in the bottom panel, Fig. 1), but only when Nile Red was mixed with $DC_{23}PC$ before the lipid was subjected to the temperature regimen which resulted in tubule formation. Some liposomal material is present in the preparation, although most of the fluorescence is associated with tubules.

A similar experiment was performed with pyrene, which has been shown to transfer rapidly between phospholipid vesicles (11). Vesicles were prepared from POPC and pyrene at a concentration of ~0.04 mol of pyrene per mole of lipid. The fluorescence spectrum of pyrene at this concentration in the POPC vesicles shows a broad emission peak at 480 nm, indicating the presence of pyrene excimer. The formation of pyrene excimer is a function of local pyrene concentration. Transfer of pyrene out of the POPC vesicles can be followed by the decrease in the ratio of excimer to monomer by measuring the relative intensity at 480 nm compared with 395 nm. Before the addition of liposomes or tubules, the excimer/monomer ratio of pyrene in POPC vesicles was 0.51. Upon mixing these pyrene-containing vesicles with ~ 12-fold molar excess of $DC_{23}PC$ lipid as liposomes, the ratio decreased to 0.08 within 3 min, indicating a decrease in the microscopic pyrene concentration from 4 to 0.2% pyrene per mole lipid. However, when pyrene-containing POPC vesicles were mixed with the same concentration of $DC_{23}PC$ lipid in the form of tubules, the ratio dropped to only 0.31, which is equivalent to a 1.7-fold decrease in the microscopic pyrene concentration. This amount of change is explained by the likely possibility of 6% of the total lipid in the preparation being in the form of liposomes instead of tubules. Thus, even pyrene, a small polyaromatic hydrocarbon, is unable to enter the lipid matrix of tubules.

Because fluorescent lipophiles cannot be inserted into preformed tubules by transfer, they must be added to the $DC_{23}PC$ lipid in chloroform before the hydration and cooling regimen required for tubule formation. As described in Materials and Methods, each fluorescent lipophile was added to $DC_{23}PC$ lipid at a concentration of 1 mol%. This level did not appear to have an adverse effect on subsequent tubule formation, and so we have used these fluorescent lipophiles incorporated into tubules as probes of tubule behavior and microenvironment.

The failure of transferable lipophiles such as pyrene to

be accepted from the aqueous phase into the lipid environment of preformed tubules suggests that the microenvironment of tubules is quite different from that of other lipid environments such as liposome bilayers. Perhaps tight packing of tubule lipid acyl chains presents a barrier to incorporation of extrinsic molecules from the aqueous phase. A probe which is sensitive to solvent viscosity may indicate a difference in packing characteristics of $DC_{23}PC$ tubules which do not accept aqueous phase lipophiles, and $DC_{23}PC$ liposome bilayers which do accept lipophiles from the aqueous phase. One such probe is laurdan, which is a sensitive indicator of polarity and solvent relaxation (12).

Laurdan is anchored in lipid bilayers by a 12-carbon acyl chain. Similar naphthalene-based compounds have been applied to studies of the phase state of bilayers (13, 14). A large charge separation in laurdan and similar molecules is the basis of their sensitivity to viscosity. The formation of the excited state is accompanied by a very large change in dipole moment. Solvent relaxation around the excited state occurs at a rate which is influenced by the microviscosity of the environment. The extent of solvent relaxation which occurs during the excited state lifetime is what determines the emission wavelength and the width of the emission spectrum. Emission spectra of such compounds are shifted to lower wavelengths when solvent relaxation is slow, such as in rigid environments, and shift to longer wavelengths when solvent relaxation is fast, as in fluid membranes. The width of the spectrum is an indication of the relationship between the rate of reorientation of solvent molecules around the new excited state dipole and the excited state decay rate. Thus, narrow spectra are expected when the fluorescence lifetime is very fast or very slow with respect to the rate of solvent relaxation.

Laurdan was added at a level of 1 mol% to $DC_{23}PC$ to permit comparison of the rigidity of the microenvironment of $DC_{23}PC$ lipid in tubules with $DC_{23}PC$ lipid as liposome bilayers. Fig. 2 shows the emission spectra of laurdan in tubules, in $DC_{23}PC$ liposomes, and in DSPC liposomes. Spectra were recorded at 20°C which is below the phase transition temperature of $DC_{23}PC$, and so for comparison, liposomes containing laurdan were prepared from DSPC, which is also below its phase transition temperature at 20°C. Laurdan in DSPC liposomes has an emission maximum at 442 nm, and a relatively narrow spectrum. The emission maximum of laurdan in $DC_{23}PC$ liposomes is 472 nm, a 30-nm shift to a longer wavelength, indicating that laurdan in $DC_{23}PC$ is in a more fluid microenvironment. However, the large width of the spectrum indicates incomplete solvent relaxation, and in fact, two distinct components may be present. The spectrum suggests the presence of at least two populations of laurdan in $DC_{23}PC$ liposomes, where some laurdan is

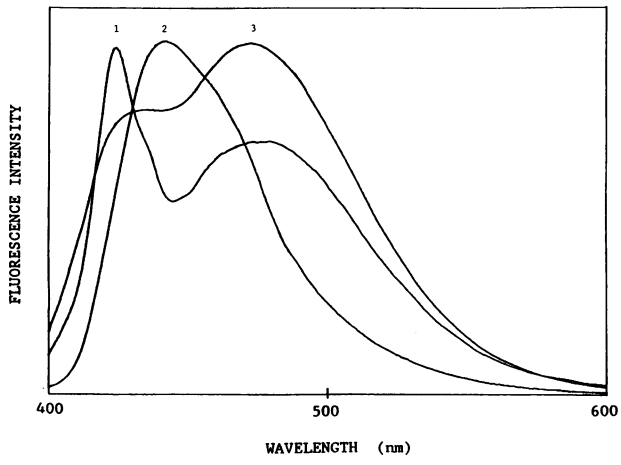

FIGURE 2 The fluorescence spectra were recorded for laurdan in $DC_{23}PC$ tubules (1), and in liposomes prepared from DSPC (2) and $DC_{23}PC$ (3). Excitation wavelength was 370 nm.

present in a relatively fluid environment (emission maximum at 472 nm) where more complete solvent relaxation is occurring before emission, and another population (emission maximum at ~423 nm) is present in a relatively rigid environment, experiencing incomplete solvent relaxation. Interestingly, the spectrum of laurdan in tubules is very similar to its spectrum in $DC_{23}PC$ liposomes, but the relative contributions from the two populations is reversed. In tubules, laurdan has an emission maximum and a very distinct peak at the shorter wavelength, suggesting a marked increase in environmental rigidity of $DC_{23}PC$ lipid in tubules compared with liposomes.

The inability of the tubule matrix to accommodate lipophiles from the aqueous phase and the spectral shift seen for laurdan in tubules suggests that perhaps the lipid packing in tubules is tighter than in other lipid matrices. To further evaluate the molecular environment of the tubule matrix, diffusion of the fluorescent lipophile, NBDPE, was examined in tubules. NBDPE has been used in photobleaching experiments by other investigators (15) to measure lateral mobility of phosphatidylcholine bilayers above and below their phase transition temperatures. Tubules were prepared from mixtures of NBDPE (at 1 mol% of total lipid) and $DC_{23}PC$ in solvent as previously described. Using fluorescence microscopy, ~50 μm of the end of an ~100 μm long tubule containing NBDPE was optically isolated by stopping down the iris of the illumination light. This area was irradiated for 5 min with unattenuated excitation light between 450 and 480 nm, causing photobleaching of the NBDPE. The result is shown in Fig. 3. The fluorescence intensity of this area was bleached to ~33% of the initial intensity. The aperture was then opened, and the field was exposed to

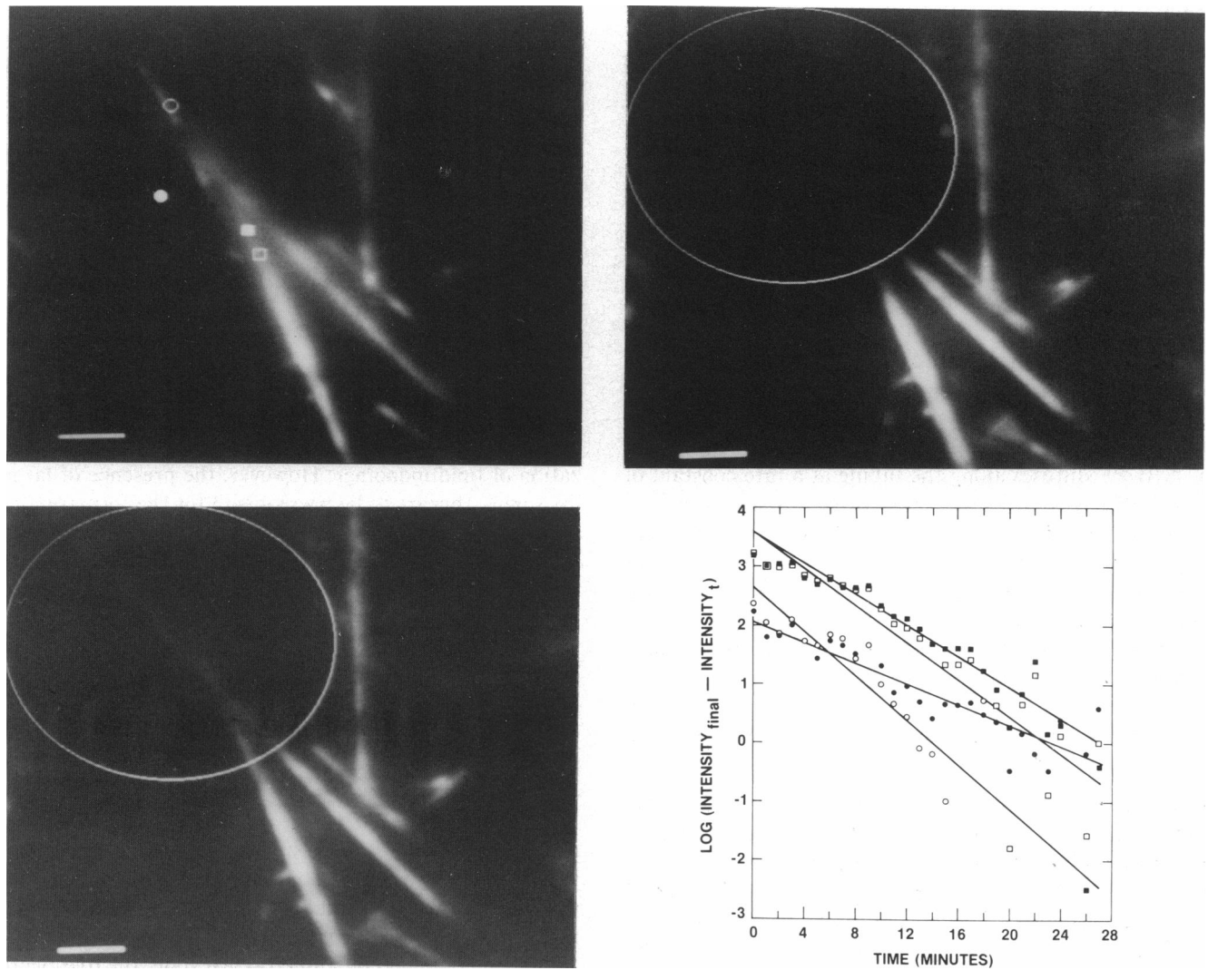

FIGURE 3 Photobleaching of NBDPE in a tubule occurred during 5 min of continuous exposure to unattenuated excitation light using the same filter set described in Fig. 1. The aperture was closed down to allow illumination of an area of ~50 μm in diam as indicated. Fluorescence recovery was monitored every minute for 30 min by 1 s exposures to 80% attenuated excitation light. Images were digitized with a Grinnel image processor. (*Upper left*) Tubule containing NBDPE before photobleaching. Intensity values of 25 pixels under the four overlay designations were analyzed in each of the 30 sequential images; (*Upper right*) after photobleaching, $t = 0$; (*lower left*) $t = 30$ min; (*lower right*) semilog plots of the increase in intensity of the designated areas.

80% attenuated excitation light for 1 s periods at intervals of 1 min for 30 min. During the excitation periods, 32 video images of the field were digitized, averaged, and stored. No detectable photobleaching of NBD occurs under these conditions.

After 30 min, the fluorescence intensity of the bleached area had increased to only 46% of the initial intensity. This increase, which is ~20% of the increase required for complete recovery, was uniformally distributed along the length and in the background. This was determined by analysis of the fluorescence intensity of three areas, each consisting of 25 pixels corresponding to 1.5 μm^2 on the bleached portion of the tubule as indicated in Fig. 3. The centers of these areas are 2, 6, and 30 μm from the edge of the unbleached area. The pixel intensities of these areas were analyzed in the 30 sequential images that were collected during the recovery period. The results of this analysis are shown in Fig. 3. All three points on the tubule display similar kinetics for the increase in fluorescence intensity. First order rate constants calculated by least squares regression analysis from the inverse of the change in intensity with time are 0.36 min^{-1}, 0.31 min^{-1}, and 0.43 min^{-1} for the indicated points on the tubule from the edge of the photobleached area to the center. A fourth

point which is not on the tubule also showed similar kinetics (0.20 min^{-1}). The similarity in these rate constants suggests that this increase in intensity is not due to diffusion of the probe but is due to spontaneous recovery of bleached fluorophores.

If fluorescence recovery occurred by diffusion of NBDPE molecules from the unbleached portion of the tubule into the bleached portion, the fluorescence intensity would increase faster in areas closer to the unbleached area. The rate of photobleaching recovery due to diffusion of NBDPE in phospholipid membranes has been reported to range from 4×10^{-8} to 5×10^{-10} cm^2 s^{-1} (15). We modeled photobleaching recovery for diffusion constants of 1×10^{-8}, 1×10^{-9}, and 1×10^{-10} cm^2 s^{-1}. Fig. 4 shows the fractional change in fluorescence intensity that is expected at each of three spots along the tubule if NBDPE diffuses along the tubule at a rate constant of 1×10^{-9} cm^2 s^{-1} (rate constant *a*) or 1×10^{-10} cm^2 s^{-1} (rate constant *b*). In Fig. 4, the theoretical photobleaching recovery curves are identified by the distance, *X*, that the spot on the tubule is from the unbleached area. Subscripts indicate the rate constant being applied.

Intensity values were calculated for one-dimensional diffusion using the expression

$$\frac{I}{I_o} = 1 - \left(\frac{2}{\sqrt{\pi}} \int_0^{x/2\sqrt{kt}} e^{-x'^2}\, dx'\right) = erfc\,(x/2\sqrt{kt}),$$

which assumes that the concentration of probe in the unbleached area is not significantly decreased, and that the tubule is infinately long with respect to the concentration gradient of the diffusing species. This second assumption is not valid when a diffusion coefficient of 1×10^{-8} cm^2 s^{-1} is used because the intensity increase at the far end of the tubule would be too great to be considered insignificant. The ratio of fluorescence intensity, *I*, to initial intensity, I_o, at the three areas indicated on the tubule is plotted as a function of time for diffusion coefficients of 1×10^{-9} and 1×10^{-10} cm^2 s^{-1}. Using the faster rate constant, fluorescence recovery at distances of 2 and 6 μm from the border of the bleached area is expected to reach ~90 and 75% of initial intensity, respectively. Recovery should reach 10% of maximum at 30 μm into the tubule. Even if diffusion were as slow as 1×10^{-10} cm^2 s^{-1}, the intensity at the 2-μm spot is expected to increase to ~75% of the prebleached value after 30 min. This compares with the 20% of complete recovery which was observed at all points. The failure of NBDPE to diffuse in the tubule during the time course of the experiment suggests that diffusion of lipid molecules in the tubule matrix is extremely inhibited.

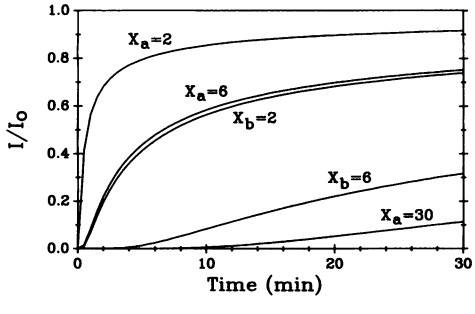

FIGURE 4 Theoretical plots of diffusion of NBDPE in tubules. The ratio of fluorescence intensity during recovery to initial fluorescence intensity is plotted for distance, *X*, along the tubule where $X = 2$, 6, and 30 μm; diffusion coefficient $a = 1 \times 10^{-9}$ cm^2 s^{-1}, and $b = 1 \times 10^{-10}$ cm^2 s^{-1}.

Considering the rigidity of the tubule matrix, it is tempting to suggest that tubules are the result of crystallization of lipid monomer. However, the presence of large liposomes appears to be prerequisite for the formation of tubules, and freeze fracture electron micrographs (2) showing the presence of several to many fracture layers, imply that tubules may be formed by the rolling up of liposomes on one another.

We studied the relationship between liposomes and tubules with fluorescence microscopy by observing tubule formation in a mixture of $DC_{23}PC$ liposomes that contained different fluorophores. Two sets of liposomes were prepared, one containing 1 mol% NBDPE in $DC_{23}PC$ and the other containing 1 mol% DiI(3) in $DC_{23}PC$. Both NBDPE (16) and DiI(3) (17) have been shown to be nontransferable between hydrophobic compartments, which means that they remain with the liposomes with which they were originally associated. The $DC_{23}PC$/fluorophore mixtures were prepared separately at 60°C, and when combined on a microscope slide, the dyes were clearly associated with two distinct populations of liposomes. As the stage was cooled from 60° to ~35°C, tubules formed. The first tubules to form contained only NBDPE fluorescence, and DiI(3) fluorescence was present only in liposomal material. After ~3 h, however, all tubules were found to contain both NBDPE and DiI(3) (Fig. 5). Images were digitized using filters which allow detection of either DiI(3) fluorescence or NBDPE fluorescence. Analysis of the correlation of pixel intensities in these two images provided a correlation coefficient of 0.82, indicating quantitatively the simultaneous occurrence of the two probes.

DISCUSSION

We have shown that formation of lipid tubules from $DC_{23}PC$ is not prevented by the presence of 1 mol% of lipid impurities. The ability to incorporate additional molecules into $DC_{23}PC$ tubules may have important

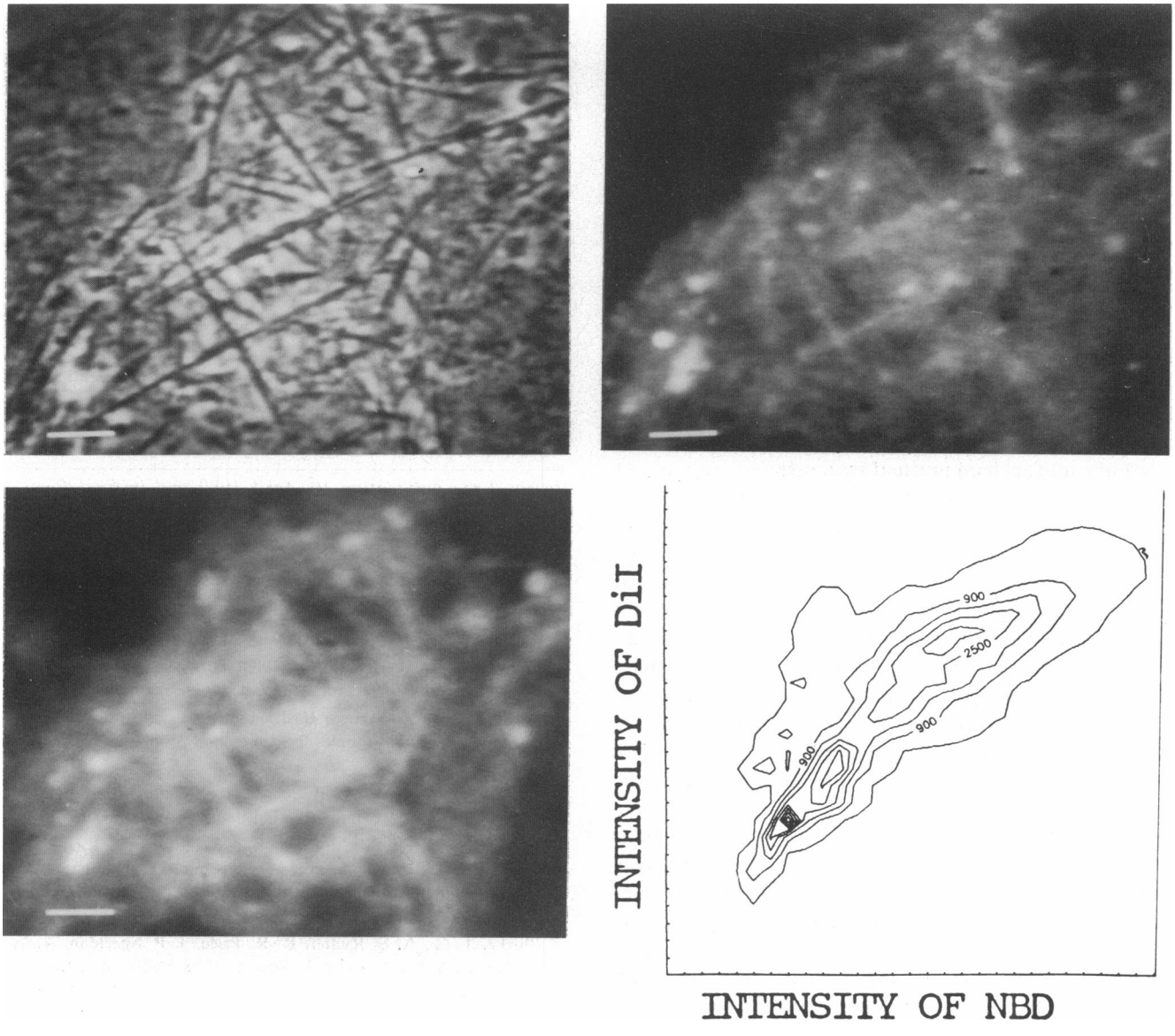

FIGURE 5 Liposomes containing $DC_{23}PC$ and either DiI(3) or NBDPE were prepared separately, combined on the microscope stage at 60°C, and allowed to cool to ~35°C. Approximately 3 h after formation of NBDPE tubules, tubules were observed to be labeled with both fluorophores. Micrographs were taken at ~35°C. (*Upper left*), Phase image of tubule preparation; (*upper right*), NBDPE fluorescence; (*lower left*), DiI(3) fluorescence; (*lower right*), three-dimensional plot of pixel by pixel correlation of NBD fluorescence intensity and DiI(3) fluorescence intensity (correlation coefficient = 0.82). NBDPE fluorescence was observed using the filter set described in Fig. 1. DiI(3) excitation bandpass was 530–560 nm, the emission was viewed through a 580-nm cut-on filter. Temperature control at the microscope stage was imperfect, and therefore this preparation contains much liposomal material that did not form tubules.

implications for the future applications of these structures. The fluorophores examined in tubules appear to be truly solubilized in the lipid matrix as determined by their fluorescence spectra and the appearance of the tubules by fluorescence microscopy. Several different experimental approaches have been used to show that although lipid-like, the microenvironment of $DC_{23}PC$ tubules is not very membrane-like.

The lipid matrix of the $DC_{23}PC$ tubule appears to be extremely rigid and tightly packed. This conclusion is based on several criteria. The fluorescence spectrum of laurdan is blue shifted in tubules with respect to its spectrum in $DC_{23}PC$ liposomes. The shift may indicate that laurdan is in a more nonpolar environment in tubules than it is in liposomes, or that solvent relaxation is incomplete (12). Polarity is not a likely contributor to the

spectral shifts, given the accompanying spectral broadening. Lakowicz et al. (13) have shown that large changes in spectral width are due to incomplete relaxation. With similar probes in lipid vesicles, Lakowicz et al. (13) and Massey et al. (14) have correlated spectral shifts resulting from incomplete relaxation with rigidity of lipid bilayers above and below their phase transition temperatures. The failure of laurodan to achieve complete solvent relaxation within its lifetime suggests that the packing of the acyl chains is tighter in $DC_{23}PC$ tubules than in DSPC and $DC_{23}PC$ liposomes. However, it is curious that the emission spectrum of laurdan in $DC_{23}PC$ liposomes is so different from its spectrum in DSPC liposomes. The spectrum suggests that the $DC_{23}PC$ lipid provides unusual microenvironments even in liposomes, and may provide clues as to why these liposomes can be manipulated to rearrange into such an unusual structure.

The rigidity of the tubule structure is confirmed by the extremely slow rate of diffusion of NBDPE in tubules. Wu et al. (15) showed that NBDPE diffuses in egg phosphatidylcholine multilayers at 4×10^{-8} cm^2 s^{-1} at 25°C, and measured similar values for egg phosphatidylcholine/cholesterol and DMPC/cholesterol mixtures. They found that in DMPC multilayers below the phase transition temperature, diffusion rates slowed to 1.5×10^{-10} cm^2 s^{-1}. In the case of $DC_{23}PC$ tubules, which are also below their phase transition temperature, we were unable to detect diffusion within the time course of the experiment.

Because of the length of time of observation, spontaneous photobleaching recovery was observed. Whereas spontaneous recovery of bleached fluorophores is problematic to all photobleaching recovery experiments (13, 18, 19), usually the rate of diffusion-associated fluorescence recovery is so fast that measurements last only seconds or a few minutes. On that timescale, spontaneous recovery is usually negligible. Tubules provide an excellent system for distinguishing lateral diffusion of fluorophores from spontaneous recovery, because diffusion can only occur from one direction. Diffusion occurring along the length of the tubule would be detected by different rates of change in intensity at different points on the tubule. The small amount of intensity increase that occurred, and the fact that it occurred everywhere in the field at the same rate, rules out the possibility that diffusion of NBDPE occurred in the tubule within the time course of the experiment.

Comparison with theoretical values indicates that diffusion of NBDPE in tubules is significantly $< 1 \times 10^{-10}$ cm^2 s^{-1}. The tubule environment is thus an extremely rigid one. This conclusion is supported by Fourier transform infrared spectroscopy data (20, 21), which indicate that tubules are highly ordered structures.

That tubules form from layers of liposomes rather than by monomer crystallization is clearly shown by the formation of tubules containing fluorophores from each of two preparations of labeled liposomes. The formation of NBDPE tubules was followed by the appearance of tubules containing both NBDPE and DiI(3). DiI(3) and NBDPE have been shown not to exchange between lipid phases, and because DiI(3) cannot spontaneously transfer into tubules, it is concluded that DiI(3) liposomes became part of preformed NBDPE tubules.

We are extremely grateful to Dr. Louis Smith for allowing us use of his microscope and imaging equipment. Many thanks to Drs. Paul Yager and Paul Schoen for their review of the manuscript.

This work was supported by the Defense Advanced Research Program Agency.

Received for publication 10 April 1989 and in final form 6 December 1989.

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