DANSYL LYSINE: A STRUCTURE-SELECTIVE FLUORESCENT MEMBRANE STAIN?

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ABSTRACT Dansyl lysine (DL) is a fluorescent compound that has significantly higher solubility in synthetic phosphatidylcholine (PC) membranes with a low cholesterol content than it does in water or in membranes having a high cholesterol content. Its fluorescence intensity is enhanced at least 50-fold when dissolved in PC membranes. Therefore, membranes with mole fractions of cholesterol (X_{ch}) $\leq$ 0.2–0.3 are stained by aqueous solutions of DL: those with a higher cholesterol content, 0.3–0.4 $\leq X_{ch} \leq$ 0.5, are not. It is proposed that DL selects for a structural feature of membranes: cholesterol-free domains. The phenomenon has provided evidence for long-lived compositional heterogeneity in large multilamellar PC-cholesterol liposomes having $X_{ch} \leq 0.2$. This is not consistent with a model in which the homogeneous state is thermodynamically favored and both intermembrane transfer and transmembrane transfer (flip-flop) of cholesterol are fast. These studies are of potential importance for understanding cell membrane structure, in particular lipid-phase equilibria and the maintenance of compositional heterogeneity between the different membranes of cells.

Recktenwald and McConnell (1), using electron paramagnetic resonance (EPR) spectroscopy, have described partitioning of 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO), a spin label, into the membranes of large, multilamellar, PC-cholesterol liposomes. At temperatures at and below the principal thermotropic phase-transition temperature (T_c) of the PC, and $X_{ch} \leq 0.2$, they note a linear decrease in TEMPO uptake with increasing X_{ch}. At $0.2 \leq X_{ch} \leq 0.5$, TEMPO uptake is low and constant. When $T > T_c$, the linear decrease range shifts to somewhat higher values of X_{ch}, so that the breakpoint is closer to $X_{ch} = 0.3$ when $T = T_c + 15$°C. Recktenwald and McConnell (1), suggest that the phenomenon reflects fluid-solid immiscibility at $T < T_c$ and fluid-fluid immiscibility at $T > T_c$, when $X_{ch} \leq$ 0.2–0.3. The concept of fluid-solid "ordered, microscopic phase separation" (2) at $T < T_c$, and $X_{ch} \leq 0.2$, was previously introduced by McConnell and colleagues (2, 3), based on extensive experimental evidence. The model describes alternating domains of pure PC and PC-cholesterol with long-range order. For the EPR studies (1), it is suggested that the spin-label selectively dissolves in pure PC (cholesterol free) domains that only occur at low cholesterol content. Evidence for preexistence of these features, rather than their induction by the probe, is provided by freeze-fracture studies in the absence of TEMPO (2). As the profile of TEMPO uptake with X_{ch} changes little on passing through T_c, the presence of cholesterol-free domains is suggested, at low cholesterol content, both above and below T_c. One particularly interesting aspect of this finding is that it is an example of a membrane phenomenon, controlled by cholesterol content, which is not related to membrane fluidity per se (i.e., as determined by the rate of lateral diffusion). As X_{ch} is raised from 0 through 0.2–0.3, the rate of lateral diffusion is decreased when $T > T_c$, but increased when $T < T_c$ (4). This paper describes the behavior of a fluorescent molecule that exhibits solubility properties similar to those of TEMPO when introduced to PC-cholesterol liposomes. Unlike TEMPO, it is capable of providing information at the single liposome (or single cell) level. This represents a distinct advantage it possesses, relative to the spin label, for certain applications.

Lipids and liposome preparation were as previously reported (5). In brief, except where otherwise noted, lipids were dried from stock ethanolic solution, then redissolved in $CHCl_3$ and dried again on a rotary evaporator. The dried lipids were suspended in phosphate buffered saline (PBS), pH 7.2, at a total lipid concentration of 10^{-3} M. For microscopic examination, the liposome preparation typically was mixed with an equal volume of stock DL solution (10^{-3} M in PBS).

Staining was rapid, and aqueous DL provided very little background fluorescence. Addition of dimyristoylphosphatidylcholine (DMPC) liposomes was capable of increasing the fluorescence intensity of DL at least 50-fold, as measured in a hemocytometer using a Zeiss Universal microscope photometer (Carl Zeiss, Inc., Thornwood, NY) with

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liposomal lipids at ~0.3M (or absent) and DL at 5×10^{-4} M. Excitation was at 365 nm and emission was >395 nm. Using a filter monochromator, we measured the λ_{max}^{em} for DL in DMPC and in PBS to be 515 and 545 nm, respectively.

DMPC liposomes readily absorb DL from its aqueous solutions, DMPC liposomes with $X_{ch} = 0.5$ do not. From data obtained using a Baird-Atomic spectrofluorometer (Baird Corp., Bedford, MA) to assay residual DL in the supernatants of liposome-DL mixtures we calculate a partition coefficient, ~10 for DMPC, and ≪1 for DMPC with $X_{ch} = 0.5$.

We find the use of a fluorescence activated cell sorter (FACS) analyzer (Becton Dickenson Co., Sunnyvale, CA) by far the best method for obtaining quantitative data regarding the staining of liposomes as a function of cholesterol content. More conventional fluorometric methods are less satisfactory because of problems introduced by the heterogeneous nature of liposome preparations, light-scattering effects, etc. Fig. 1 shows results that correlate with those obtained by Recktenwald and McConnell (1) using the EPR method with TEMPO. We have studied the effect of temperature on the phenomenon by microscopy. A Zeiss Universal microscope equipped for the epifluorescence technique was used with a band-pass (365 nm) excitation filter, a long-wave pass (>395 nm) barrier filter, and a heated stage. Liposomes prepared with DMPC (T_c = 23°C) and dipalmitoylphosphatidylcholine (DPPC) (T_c = 41°C) were examined. At temperatures below and close to T_c for the particular PC, liposomes with X_{ch} in the

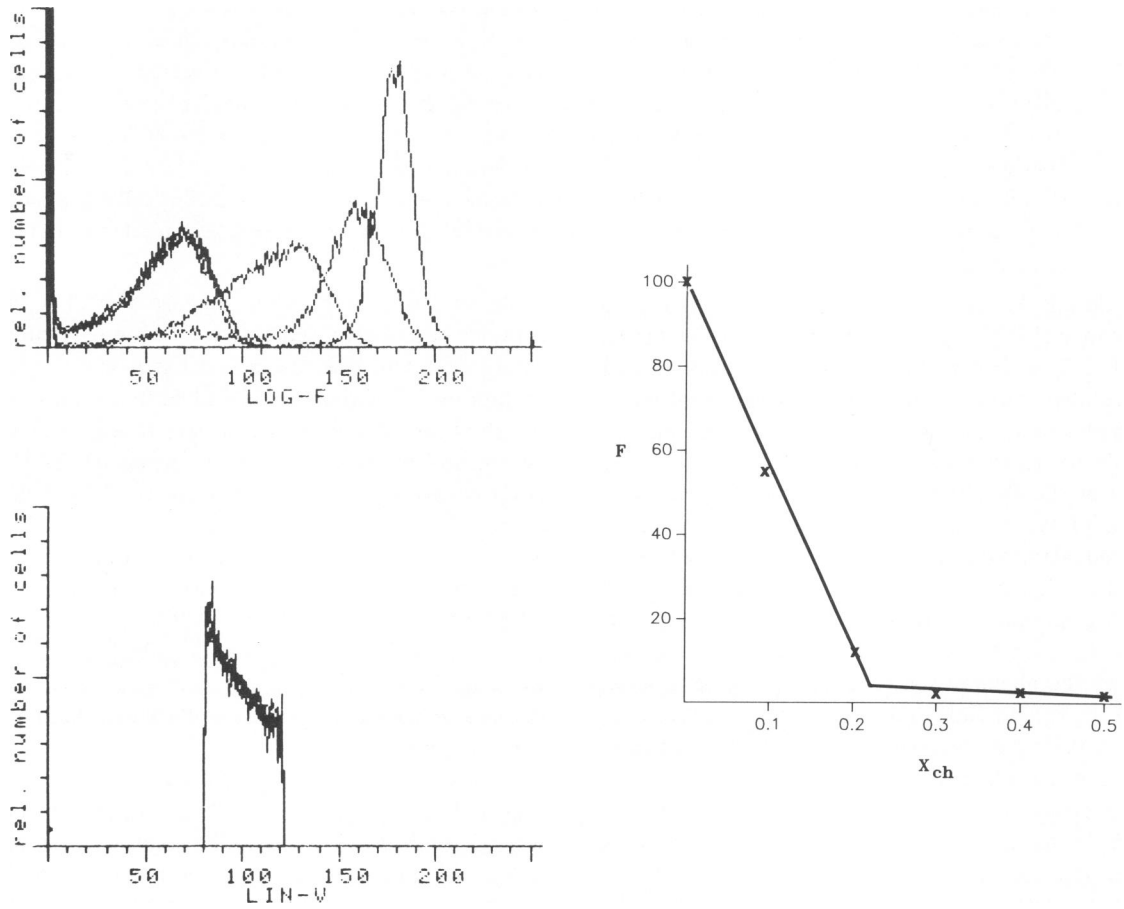

FIGURE 1 The fluorescence of sized DMPC liposomes exposed to DL, as a function of X_{ch}. Data were obtained at 21°C using a Becton Dickinson FACS analyzer. The two histograms (left side of figure) each depict six superimposed plots taken from six samples of liposomes that varied in X_{ch}. Samples were introduced in saline at 10^{-4} M in total lipid and 10^{-4} M in DL. For these particular plots, the instrument was gated to take data from 10^4 particles (called "cells" on the plots) in a restricted size range, ~5 μm diam. All six samples had large populations in this size range and, as shown by the lower histogram, their distribution profiles were exactly superimposable. The upper histogram shows fluorescence intensity on an arbitrary logarithmic scale from this size population. In this case, only the plots from liposomes having $X_{ch} = 0.3$, 0.4, and 0.5 are closely superimposable. These are on the extreme left of the upper histogram. Reading from left to right, the main peaks of the other three plots were generated from liposomes with $X_{ch} = 0.2$, 0.1, and 0. Marked heterogeneity of fluorescence is clearly shown in the $X_{ch} = 0.1$ plot. Heterogeneity is also evident in the $X_{ch} = 0.2$ plot. Similar data are generated with the instrument gated for other size ranges. The instrument was fitted with a UG1 excitation filter, a short-pass 375-nm dichroic mirror and two long-pass 400-nm emission filters. At the right side of the figure is shown a linear transform of the data from the upper histogram. On the arbitrary linear scale used for F (the total fluorescence) unstained particles give a background reading of ~1.

range 0–0.20 are stained (with decreasing intensity) by the dansylated molecules; those with higher concentrations of cholesterol are not stained. At temperatures ~15°C above T_c, the X_{ch} range compatible with staining is extended to 0.25 or 0.30. We conclude that DL selects the same membrane structural feature as does TEMPO, and that this is likely to be the presence of cholesterol-free domains. Membranes prepared with sphingomyelin in place of PC behave in the same way with DL.

By visual inspection we note marked heterogeneity of staining in some liposome preparations having $0 < X_{ch} \leq 0.2$. This is supported by the FACS analyzer data (see Fig. 1). Although, on microscopic examination, we have routinely found it easier to pick out examples of heterogeneity when lipids are dried from C_2H_5OH (as final solvent) rather than from $CHCl_3$, FACS analyzer data clearly indicates that it may occur using either method. The FACS analyzer was gated on Coulter volume, rather than scattered light, so that the gated population has very little variation in diameter. Within this population, there will be variation in the number of bilayers present. This is true for liposomes of any composition and is frequently evident on observation of liposomes by phase contrast microscopy. However, whereas for the case of liposomes with $X_{ch} = 0$, DL staining intensity is always noticeable and is roughly proportional to lipid content as judged by phase contrast microscopy, this is not true for the case of many liposomes with $0 < X_{ch} \leq 0.2$ (see legend of Fig. 2 for further explanation). Also, the FACS analyzer indicates a smooth distribution of fluorescence intensities around a peak occupancy value for liposomes having $X_{ch} = 0$, but frequently shows two distinct populations for the case of liposomes having $0 < X_{ch} \leq 0.2$. We conclude that the phenomenon of staining heterogeneity, as described, does not reflect a simple variation in the number of bilayers present.

The apparent stability of staining heterogeneity is surprising: presumably it reflects long-lived compositional heterogeneity. We have excluded the possibility that it is an artifact, introduced by inadequate mixing of stain and liposomes, by microscopic and FACS analyzer examination of preparations in which DMPC liposomes were added to DL as two separate aliquots 30 s apart. Heterogeneous staining is not detected under such conditions.

In preliminary experiments aimed at examining the stability of compositional heterogeneity, liposomes having a nominal $X_{ch} = 0.20$ and obviously heterogeneous profiles (as shown by stained samples) were held at 4°, 37° and 60°C for 12 d, 35 d, and 24 h, respectively (2×10^{-3} M sodium azide was included for those incubated at 37°C). No obvious change in profile was observed on microscopic examination after staining. In another attempt to observe a change in staining profile that might indicate redistribution of cholesterol, a slide with two adjacent structurally similar liposomes, showing marked heterogeneity of staining, was left on the microscope stage in the same position for 96 h at room temperature. After that time, no change could be seen by visible light or by UV illumination (see Fig. 2). We have very limited FACS analyzer data, at this time, regarding the stability of heterogeneity. What we do have does not indicate progress towards compositional homogeneity with time.

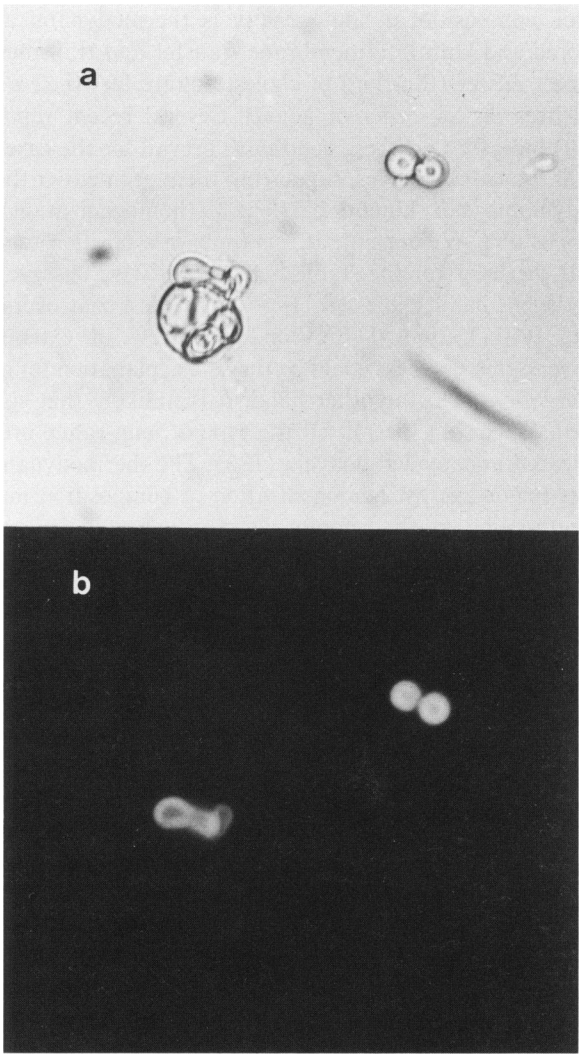

FIGURE 2 An example of heterogeneous staining. DMPC-cholesterol liposomes, with $X_{ch} = 0.20$, prepared by drying the lipids from C_2H_5OH and suspending in the usual way before mixing with dansyl lysine. Total lipids and DL were both at 5×10^{-4} M. In *a* illumination is by visible light, and in *b* by the epifluorescence technique. The central group of liposomes is ~10 μm in diameter. The robust liposome that fails to fluoresce, as shown by the 90 s Kodak Tri-X Pan (Eastman Kodak Co., Rochester, NY) photographic exposure in *b*, is also completely invisible to the eye by this type of illumination. This striking lack of a monotonic relationship between staining intensity and quantity of lipid, as judged by visible light microscopy, is quite unique to this type of preparation in our experience. It is not seen with liposomes having $X_{ch} = 0$ that are stained by DL, nor is it seen with liposomes made fluorescent by inclusion of a fluorescent lipid and having $X_{ch} = 0$–0.5, such as were used by Rubenstein et al. (4) to study the rate of lateral diffusion as a function of X_{ch}. Similar close association of a staining and a nonstaining liposome has been monitored over a 96 h period and found not to change. Details regarding the microscope are in the text.

These observations cannot be explained by a model in

which compositional homogeneity is thermodynamically favored and both intermembrane transfer and transmembrane transfer (flip-flop) of cholesterol are fast (i.e., with half-lives on the order of hours). Several recent reports (6–9) indicate that these conditions prevail for the case of small sonicated vesicles, suggesting there are neither thermodynamic nor kinetic barriers to homogenization of composition. We are not, at this time, in a position to say what the basis for the stable heterogeneity we observe is. On the one hand, cholesterol flip-flop rate is a controversial issue (6–11), and if flip-flop were slow in extended bilayers, this slowness could be the sole explanation for our observations. On the other hand, it is unlikely that sonicated vesicles are permitted the type of long-range order indicated in extended bilayers (1–3). The thermodynamic basis for or against homogenization of composition may, therefore, be quite different for the case of sonicated vesicles, as compared with that for the extended bilayers of multilamellar liposomes, or biological membranes. Staining by DL may prove a useful method for further studies designed to investigate this possibility as well as having provided additional evidence supportive of "ordered, microscopic phase separation" (1–3) in PC-cholesterol liposomes with low cholesterol content.

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