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Lateral Phase Separation of Phospholipids as a Basis for Increased Permeability of Membranes Towards Fluorescein and Other Chemical Species

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Summary. Using mouse spleen cells, before and after treatment with glutaraldehyde or mild hyperthermia, we observe a strong correlation between permeability to fluorescein and susceptibility to staining with Nε-dansyl-L-lysine (irrespective of the cells' ability to exclude trypan blue). We observe the same correlation using liposomes prepared from phosphatidylcholine and varying amounts of cholesterol. We have recently introduced Nε-dansyl-L-lysine as a fluorescent membrane stain, or "probe," whose uptake, we propose, is selective for phospholipid domains in membranes (G.M.K. Humphries & J.P. Lovejoy *Biophys. J.* **42**:307–310, 1983; G.M.K. Humphries & J.P. Lovejoy *J. Membrane Biol.* **77**:115–122, 1984). The results presented here are consistent with the hypothesis that the presence or absence of phospholipid domains in membranes also modifies their permeability toward fluorescein, and suggests that permeability towards other chemical species may be similarly affected. On the basis of work using liposomes, we believe this to be the case for carboxyfluorescein and for glucose.

Key Words phospholipid domains · membranes · fluorescein diacetate · Nε-dansyl-L-lysine · permeability

Introduction

Our recent work has supported the hypothesis that Nε-dansyl-L-lysine (DL) selectivity partitions into (and diffuses through) membranes whose component molecules have undergone lateral phase separation resulting in the formation of phospholipid (PL) domains; this has led to our introduction of DL as a fluorescent stain (or "probe") with putative selectivity for PL domains in membranes (Humphries & Lovejoy, 1983*a*,*b*).

In certain instances, changes in the functional state of biological cells parallels changes in their susceptibility to staining with DL (Humphries & Lovejoy, 1984). As selective permeability is fundamental to membrane function, it is of interest to compare the permeability of membranes towards compounds other than DL with their susceptibility to staining with that fluorescent molecule.

Fluorescein diacetate (FDA) is taken up by cells and hydrolyzed by intracellular enzymes to the more polar fluorescent molecule, fluorescein (FL). As a result, appropriately illuminated cells whose plasma membranes have low permeability to FL develop intense fluorescence shortly after exposure to FDA, because they trap FL in aqueous solution. FL does not stain the cell membranes through which it escapes. In many cases, the ability of their membranes to retain FL appears to be correlated with cell viability. This has led to the use of FDA for determination of the viability of certain types of cells (Rotman & Papermaster, 1966).

For the case of freshly isolated mouse spleen cells, viability as determined using FDA is the same as that determined by exclusion of trypan blue (TB). However, we find that mouse spleen cells modified by procedures which render their membranes susceptible to staining with DL no longer retain FL, even though they may remain resistant to staining with TB. Thus "viability" as determined using TB does not necessarily correspond to "viability" as determined using FDA. The present report describes this work and our subsequent use of a synthetic membrane system in an attempt to explain the correlation between staining with DL and permeability towards FL and other small polar molecules.

Abbreviations

DL, Nε-dansyl-L-lysine; PL, phospholipid; FDA, fluorescein diacetate; FL, fluorescein; TB, trypan blue; FCS, fetal calf serum; GA, glutaraldehyde; PBS, phosphate-buffered saline; DPPC and DMPC, L-α-dipalmitoyl- and L-α-dimyristoylphosphatidylcholine; DCP, dicetylphosphate; CF, carboxy-fluorescein; GABS, glucose assay-buffered saline; T_c, phase transition temperature; X_{ch}, mole fraction of cholesterol.

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Materials and Methods

Spleen Cells

Cells were prepared by disaggregation of spleens from 8 to 20-week-old BALB/c female mice. Red cells were removed using Ficoll-Paque (Pharmacia) and the nucleated cells suspended in RPMI (Gibco) with 5% fetal calf serum (FCS) at 2×10^7/ml. Glutaraldehyde (GA) and heat treatments were as described in the Figure legends.

Staining with DL

A stock solution of 10^{-3} M DL in phosphate-buffered saline (PBS: 0.145 M NaCl with 0.01 M phosphate, pH 7.2) was mixed with an equal volume of cell or liposome preparation prior to examination by epifluorescence microscopy or flow cytometry. Staining is very rapid. There is no need to attempt to remove unbound DL. For microscopy, excitation was filtered at 360 nm and emission at >395 nm. For flow cytometry, a Becton-Dickinson FACS analyzer fitted with a UG1 excitation filter, a short-pass 375 nm dichroic mirror and two long-pass 400 nm emission filters was used.

Loading of Spleen Cells with FL

The method of Rotman and Papermaster (1966) was used. Spleen cells were exposed to FDA at a final concentration of 6×10^{-7} M for 15 min at room temperature. This leads to uptake of FDA and intracellular hydrolysis to FL. The FL is trapped by approximately 99% of freshly prepared spleen cells, causing them to fluoresce when viewed using epifluorescence microscopy with excitation filtered at 450 to 490 nm and emission at 520 to 560 nm. For freshly prepared cells, the number failing to accumulate FL is the same as that staining with TB. Prior to GA treatment, FL-loaded cells were washed with PBS and resuspended at 2×10^7/ml in PBS.

Lipids and Liposomes

L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-dimyristoylphosphatidylcholine (DMPC) and dicetylphosphate (DCP) were purchased from Sigma. Cholesterol was purified on Sephadex LH20 as previously described (Humphries & McConnell, 1979). Multilamellar liposomes were prepared as previously described (Humphries & Lovejoy, 1983*b*) except that, prior to drying from organic solvents, 0.2 μmol DCP was added to each 10 μmol DPPC-cholesterol to increase carboxyfluoroscein (CF) or glucose trapping when those substances were to be used. Inclusion of DCP does not affect the DL-staining profile of the liposomes. Liposomes used for purposes other than the trapping/release of CF or glucose did not contain DCP.

Liposomes were generated from dried lipids at 60°C in the presence of PBS, 0.3 M glucose or 0.1 M CF (Eastman Kodak). The pH of the latter solution was first adjusted to 7.4 with NaOH. The initial liposome concentration was 10^{-2} M with respect to total lipid. The glucose- and CF-loaded liposomes were cooled on ice and washed four times with 20 volumes of ice-cold PBS. (Centrifugation was at 4°C and 1,800 $\times$ *g* for 7 min each time.) These loaded liposomes were finally suspended either in ice-cold PBS (for the CF release assay) or in ice-cold glucose assay-buffered saline (GABS: 0.15 M NaCl, 5×10^{-3} M TRIS, 3×10^{-3} M $MgCl_2$, 1.5×10^{-4} M $CaCl_2$, pH 7.5) at a concentration corresponding to 2×10^{-4} M in total lipids if loss incurred during washing is ignored. They were kept on ice until required, for a period not exceeding 6 hr.

Assay for Permeability of Liposomes Towards FL

5 μmol of each type of DPPC-cholesterol liposomes, prepared in PBS, were pelleted and resuspended in PBS with 10^{-2} M disodium FL added prior to maintenance for 1 hr at 42°C. At this stage, liposomes were at approximately 50 μmol lipid/ml and FL at approximately 5×10^{-3} M. After the heating period the liposomes were cooled on ice, mixed with 100 volumes of cold PBS, and centrifuged. The pelleted liposomes were lysed by treatment with 0.1% Triton at 75°C and the fluorescence of the resultant solutions determined. Neither the Triton, nor the lipids, interfere with the FL fluorescence at the relevant concentrations. The proportion of total FL trapped by each liposome preparation was calculated from fluorimetric data. At each centrifugation step the supernatant was clear, indicating negligible loss of lipids to the supernatant. (*See also* Fig. 4 and its legend for an additional method for observing differences in permeability of liposomes towards FL.)

Assay for Glucose Released from Liposomes

Released glucose was assayed using a modification of the method described by Kinsky and colleagues (Haxby, Kinsky & Kinsky, 1968). Hexokinase, glucose-6-phosphate dehydrogenase, NADP and ATP · Na_2 were purchased from Sigma. The enzymes were dialyzed extensively versus distilled water and stored in convenient aliquots at −20°C. The enzyme-coenzyme mixture, prepared freshly each day the assay was performed, consisted of GABS with 3×10^{-3} M ATP and 2×10^{-3} M NADP together with hexokinase and glucose-6-phosphate dehydrogenase, each at approximately 2 units/ml.

Liposomes in GABS, prepared as described in the preceding section, were maintained at the required temperature for the required length of time and then 1-ml aliquots were brought rapidly to room temperature by placing them in a water bath. Enzyme-coenzyme mixture (1 ml) was added to each sample and the OD_{340} was read after 5 min. (Preliminary experiments have shown that these conditions are consistent with a linear relationship between OD_{340} and glucose concentration, for the concentration range of interest.)

For background (ultrapped) glucose determination, liposomes maintained on ice were first brought to room temperature by immersion of tubes containing 1-ml aliquots in a room temperature water bath for about 5 min. For determination of total glucose (trapped and nontrapped), 5 μl Triton X-100 was added to 1-ml aliquots of the liposomes and they were then heated at 60°C for 10 min prior to cooling to room temperature and addition of the enzyme-coenzyme mixture. Triton, at this concentration, does not interfere with the glucose assay. Initial trapped glucose was calculated from these values. Released glucose was determined by subtracting the initial untrapped concentration level from that measured after each heating procedure was completed.

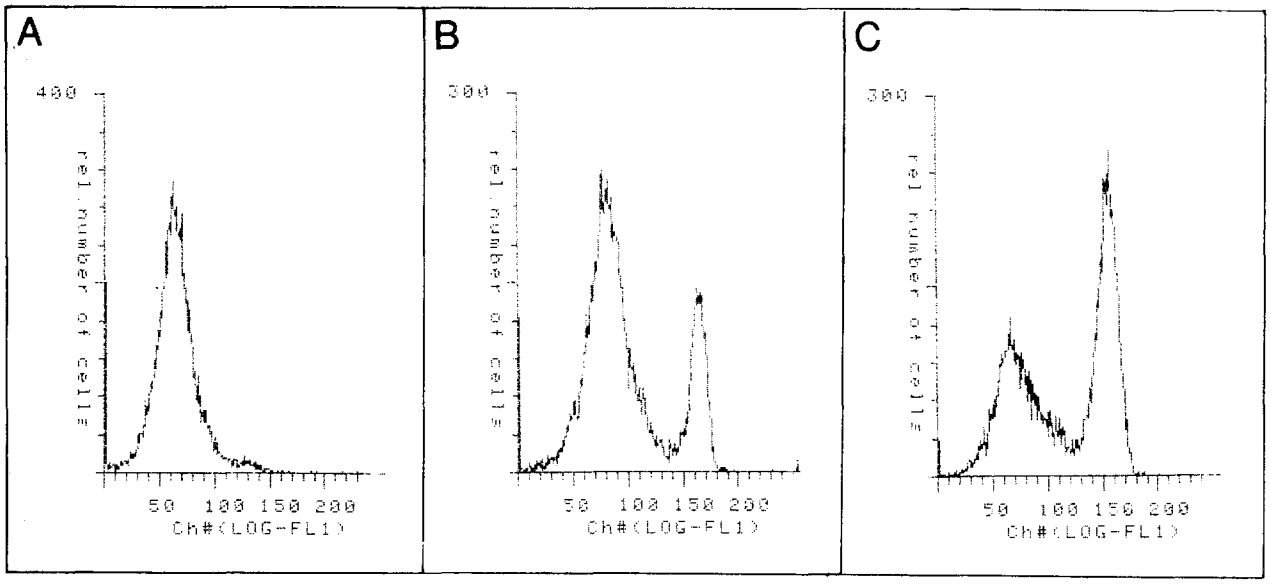

Fig. 1. Heat treatment of mouse spleen cells leads to generation of two populations, only one of which stains with DL as demonstrated by a significant increase in fluorescence relative to that given by cells in the absence of DL. The size of the staining population increases with temperature, for the fixed heating period used. Panels A, B and C show flow cytometric data (normalized to a fixed maximum peak height) from Ficoll-Paque separated spleen cells, maintained for 20 min at 37, 43 and 45°C, respectively. Cells were at 2 × 10^7/ml in RPMI medium with 5% FCS during the water bath heat treatment. After heating they were stained by mixing with an equal volume of 10^{-3} M DL in PBS. Little difference was seen in the relative sizes of the two populations when 0.1% TB was included with the DL solution (confirming our microscopic observation that few cells fail to exclude TB under these conditions). However, TB shifts the fluorescence of the staining population to a slightly lower intensity, presumably because of a quenching effect in the medium or at the surface of the cells (*data not shown*). Please see text for further discussion

Assay for CF Released from Liposomes

The fluorescence of 0.1 M CF trapped in liposomes is markedly self-quenched (Weinstein et al., 1977). Release of the compound was monitored by measuring the increase in fluorescence after each heating procedure was completed, using a simple fluorometer with excitation filtered at 495 nm and emission at >510 nm. Total trapped CF was determined by lysing the liposomes with Triton, as for the glucose-loaded preparations, and subtracting fluorescence contributed by unlysed, unheated liposomes. For the relevant values, the fluorescence of released CF is linear with concentration. FL cannot be used in an analogous manner because it destabilizes liposomes when used at a high concentration.

Results and Discussion

In a previous report (Humphries & Lovejoy, 1984), we showed that spleen cells treated with GA (0.05% for 15 min) or heat (41 to 45°C for 20 min) develop susceptibility to staining with DL. The GA-induced, DL-staining, TB-excluding state is generally more stable than the heat-induced, DL-staining, TB-excluding state. In the latter case, exposure to low ionic strength buffers, or buffers lacking FCS, leads to a rapid change in most of the DL-staining cells to produce the TB-staining state. In contrast, GA-treated, DL-staining cells maintain the TB-excluding characteristic for at least 1 hr in low ionic strength buffers or those without FCS. The dose response to both moderate hyperthermia and GA treatment is an increase in the number of cells in a staining population and a corresponding decrease in the number of cells in a nonstaining population: i.e., by FACS analyzer examination, the response to both types of treatment is "all-or-none." This is illustrated by Fig. 1 for the case of heat treatment. Similar plots are obtained using GA at low concentrations; Fig. 2 includes data taken from such plots. In both cases, the nonstaining population (e.g., left-hand side of each plot in Fig. 1) has a mean fluorescence intensity peak very little greater than that of cells to which DL has not been added (*see also* Humphries & Lovejoy, 1984) and the staining population (right-hand side of each plot in Fig. 1) has a mean fluorescence intensity which is approximately constant (and has the same value after heat treatment or GA treatment). Certain types of spleen cells may be more susceptible than others to heat and/or GA treatment.

Figure 2 also shows the release of FL from GA-treated, FL-loaded spleen cells. Under the conditions used, FL release is clearly GA dose-dependent, and parallels the percentage of cells staining

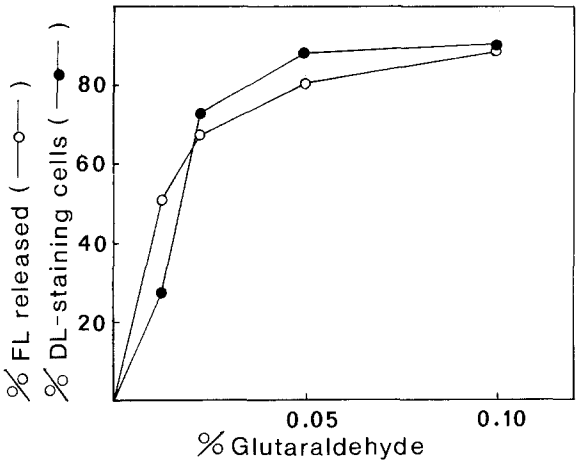

Fig. 2. GA treatment causes release of FL from FL-loaded mouse spleen cells (–○–). Cells, prepared as described in the text, were exposed to GA at the concentrations indicated, for 15 min at 22°C, with gentle shaking. They were then centrifuged and the supernatants removed for fluorometric assay. A value for total lysis was obtained by freeze-thawing cells in liquid N_2. All data was corrected for spontaneous release of FL from cells exposed to 0% GA. Also shown is the percentage of cells which stain with DL as determined using the FACS analyzer (–●–). Figures were corrected for 4% DL-staining cell present in the control preparation exposed to 0% GA

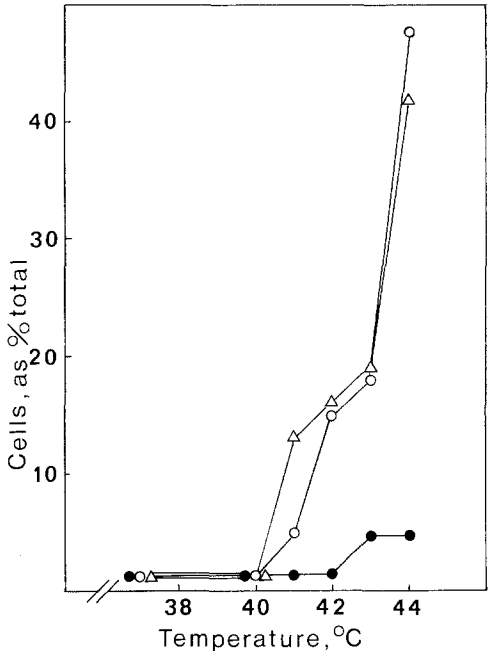

Fig. 3. The staining profile of Ficoll-Paque-separated mouse spleen cells after heating for 20 min at the temperatures shown. Cells, at 2×10^7/ml in RPMI medium with 5% FCS, were heated in the water bath, cooled to room temperature, and aliquots stained by mixing with equal volumes of 10^{-3} M DL in PBS, 0.1% TB in PBS, or 4.8×10^{-5} M FDA in PBS (prepared by dilution of a stock 5 mg/ml acetone solution). DL- and TB-staining cells were counted immediately. Cells stained by addition of FDA (i.e., those accumulating FL) were counted after 10 to 15 min. The graph shows the percent of DL-staining cells (–○–), TB-staining cells (–●–) and cells which failed to stain on exposure to FDA (–△–). 250 to 1000 cells were scanned to provide each data point. For observation of DL, excitation was filtered at 360/10 nm and emission at >395 nm. For observation of FL, excitation was filtered at 450 to 490 nm and emission at 520 to 560 nm. (FDA sticks to glass hemocytometers and cover slips. If it is not totally removed by hot water and detergent between counts it can give misleading results: FL-stained cells are often visible using the filter set used for observation of DL-stained cells.)

with DL. As previously noted, GA-treated membranes are intact by the criterion of TB exclusion.

Figure 3 shows that the proportion of cells which stain with DL, after heat treatment, is the same as that which fails to stain with FDA. In contrast, the proportion of cells which stains with TB is far smaller than that which stains with DL, as previously reported. Cells which stain with TB also stain with DL. If DL and TB are used together, the DL-staining of cells which fail to exclude TB is quenched (Humphries & Lovejoy, 1984). Failure of a population of heat-treated cells to stain with FDA could be based on (a) failure of heat-treated membranes to take up FDA, (b) failure of heat-treated enzymes to hydrolyze FDA to FL, or (c) failure of heat-treated membranes to retain FL. The first of these possibilities is unlikely because heat-treated cells, but not their isolated supernatants, rapidly hydrolyze FDA to FL which is retrievable from the cell supernatant, suggesting that the FDA *does* have access to the cell-associated enzymes. The second of the possibilities is unlikely because a cell extract, exposed to 44°C heat treatment for 20 min, hydrolyzed FDA at a rate little different from that of a nonheated extract. By process of elimination, the third possibility, that the membranes of some heat-treated cells fail to retain FL, seems the most probable explanation. This model is also supported by fluorometric analysis of the supernatants of heat-treated cells, preloaded with FL. (*data not shown*).

The correlation between the spleen cells' susceptibility to staining with DL and their permeability towards FL suggests a common basis. We have previously proposed that DL staining is selective for PL domains in membranes and that such domains are likely to form after treatment of biological membranes with agents capable of cross-linking or aggregating membrane proteins: the rationale is that concomitant release of protein boundary lipids rich in PL could lead to PL domain formation (Humphries & Lovejoy, 1984).

Our previous work did not support the possibility that aggregation/cross-linking of membrane proteins was a *direct* cause of DL staining because the molecule shows little interaction with native proteins and even less with aggregated proteins (Humphries & Lovejoy, 1984). However, this does not preclude the possibility that aggregated membrane proteins could be a direct cause of increased permeability towards FL, and this still remains a possibility. In order to address the question as to whether or not permeability towards FL is likely to increase as an *indirect* consequence of protein aggregation (i.e., is it facilitated by the presence of PL domains?) we have turned to examination of a synthetic membrane system employing DMPC or DPPC and cholesterol, but no proteins.

Either cholesterol or protein, included in synthetic PC membranes, similarly suppresses cooperative melting at the principal thermotropic phase transition temperature (T_c) of the PC (and other physical demonstrations of PC molecular cooperativity), suggesting that PC-PC interactions are replaced by PC-cholesterol or PC-protein interactions (Owicki & McConnell, 1979; Pink & Chapman, 1979). Recent work by McConnell and colleagues (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Recktenwald & McConnell, 1981) and by others (Presti, Pace & Chan, 1982) has led to theoretical models for cholesterol-synthetic PC bilayer structure which have many similar features. Long-range molecular ordering is proposed, with elongated microdomains of cholesterol-rich lipids alternating with elongated microdomains of pure PC. Copeland and McConnell (1980) describe this as "ordered, microscopic phase separation." X-ray diffraction studies of Hui and He (1983) are also supportive of such models. A low mole fraction of cholesterol (X_{ch}), ≤ 0.2, is required for PC domains to manifest their presence through physical demonstration of cooperativity. In this composition range, we find that DPPC and DMPC liposomes take up DL and are stained by it, as determined using fluorescence microscopy or flow cytometry. At higher cholesterol content, $0.3 \leq X_{ch} \leq 0.5$, liposomes are not stained by DL nor do they exhibit domain formation, as shown by a lack of PC cooperativity. We have, therefore, proposed that DL uptake and staining is specific for PC (and various other PL) domains in membranes (Humphries & Lovejoy, 1983*b*).

It should be stressed that our evidence indicates that DL does not recognize PL molecules *per se*, but some feature characteristic of their gel or liquid crytalline array when a multiplicity of certain PL molecules form a complete bilayer, or a domain within a bilayer. We have no information as to the exact site(s) recognized by DL or the orientation of bound DL with respect to the bilayer.

Of primary interest, with relevance to heat- and GA-treated cell permeability, was the question as to whether or not liposomes which were susceptible to staining with DL (i.e. $X_{ch} \leq 0.2$) were more permeable to FL than those which were resistant to such staining ($0.3 \leq X_{ch} \leq 0.5$). A simple demonstration that this *is* the case is illustrated by Fig. 4. An analogous result is obtained by replacing liposomes having $X_{ch} = 0$ by GA-treated spleen cells, and liposomes having $X_{ch} = 0.5$ by untreated spleen cells. These results are also obtained using CF in place of FL but permeation is slower for CF. Figure 5 shows that influx of FL shows a break between $X_{ch} = 0.2$ and $X_{ch} = 0.3$, as is the case for staining with DL (Humphries & Lovejoy, 1983*b*), and provides further evidence that permeability to FL parallels susceptibility to staining with DL. Experiments similar to those shown in Fig. 4 and Fig. 5 have shown that permeability to FL is greater at and above T_c for the PC than it is below.

It has been shown that the molar surface area of multilamellar liposomes is not affected by X_{ch} (Schwartz & McConnell, 1978). Thus the phenomena illustrated by Fig. 4 and Fig. 5 cannot be explained on the basis of liposomes with $X_{ch} = 0.5$ having more bilayers than those with $X_{ch} = 0$. We conclude that membranes having fluid PL domains are highly permeable to FL, whether or not membrane proteins are included. The high permeability of heat and GA-treated spleen cells towards FL could, therefore, be explained by the presence of PL domains. (It is anticipated that these would be fluid for the case of spleen cell membrane PL.)

Microscopic examination of cells and liposomes which stain with DL, makes it clear that the compound stains and passes through the outermost membrane and then stains internal structures, including membranes, as well. In contrast, FL does *not* appear to stain the membranes through which it passes. Presumably this difference in behavior between FL and DL is based on differences in their partition coefficients and the level of fluorescence enhancement they experience in hydrophobic environments.

If DL *does* recognize PL domains in plasma membranes, then cells which stain with DL may be generally permeable to substances which readily permeate PL domains in PC-cholesterol liposomes. (Although the converse is not necessarily true: differences between cholesterol and proteins, protein-mediated transport etc. must be considered.) Several previous studies by others have shown that cholesterol suppresses the permeability of a variety of small polar chemical species, including glucose,

K^+, Na^+ and Cl^- (Demel, Bruckdorfer & Van Deenen, 1972; Papahadjopoulos, Nir & Ohki, 1972). However, these studies typically used egg lecithin, a PC which, we find, fails to demonstrate sharp changes in phase behavior at $X_{ch} \simeq 0.2$ by the criterion of DL-staining. DL-staining of egg lecithin liposomes is roughly inversely proportional to cholesterol content, as is their reported permeability to glucose, etc.

As a means of critically examining our hypothesis, we were interested in seeing whether it was possible to detect a sharp break in permeability of liposomes at $X_{ch} \simeq 0.2$, if a synthetic PC were used in place of egg PC. Figure 6 illustrates an experiment showing this to be the case for glucose. We have obtained analogous results using CF (*results not shown*). It is of interest that permeability is greater at $X_{ch} \leq 0.2$ than at $0.3 \leq X_{ch} \leq 0.5$, both

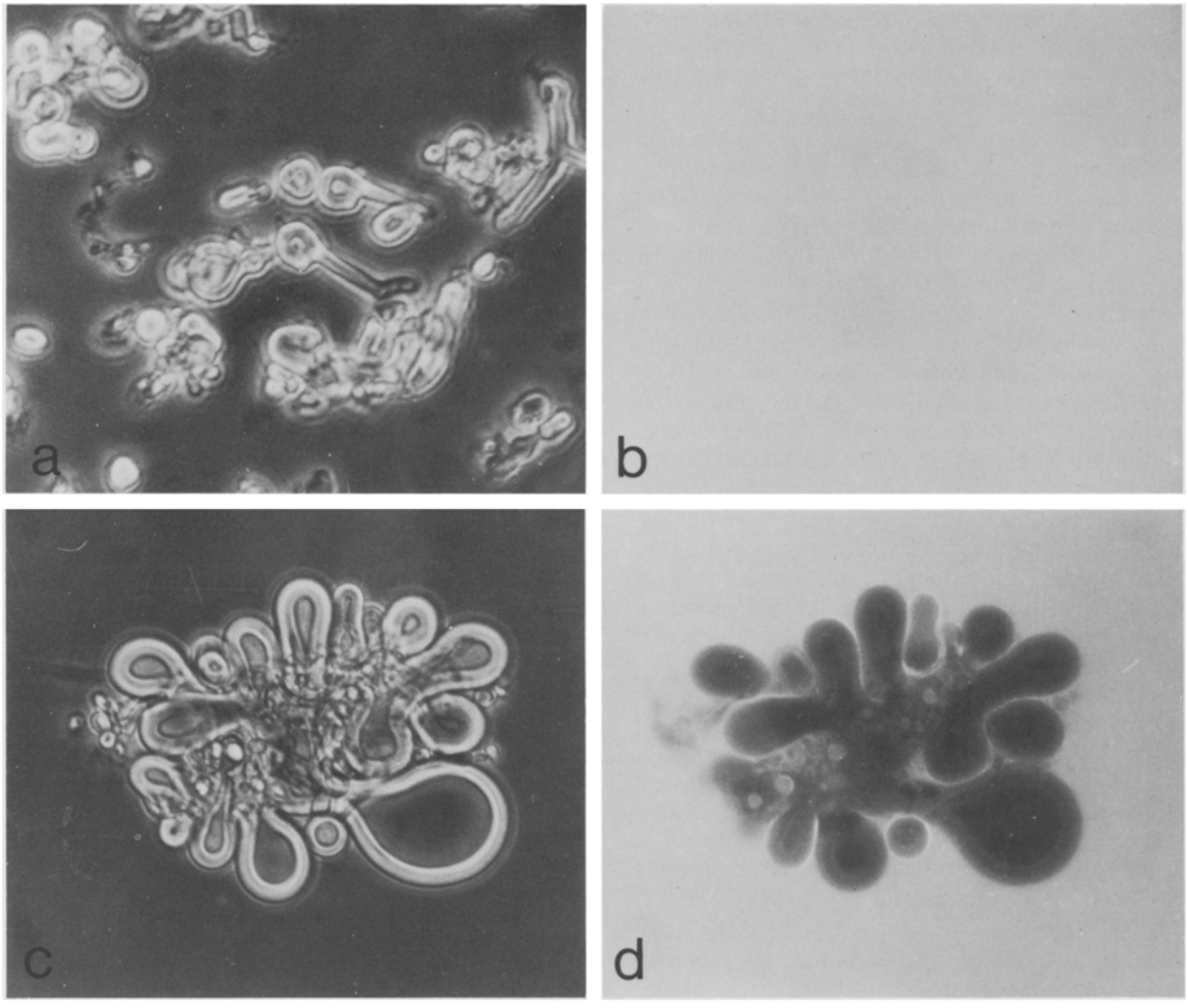

Fig. 4. Permeability of liposomes towards FL. Liposomes were prepared in PBS and then mixed with an equal volume of PBS containing 10^{-5} M FL. They were then heated for 20 min at 30°C. 4*a* and 4*c* show DMPC liposomes, with $X_{ch} = 0$ and 0.5, respectively, by phase contrast microscopy. 4*b* and 4*d* show the same fields using the epifluorescence technique, with excitation filtered at 450 to 490 nm and emission at 520 to 560 nm. Immediately after mixing, both types of liposomes give rise to the "black hole effect," as shown in 4*d*, presumably because they exclude FL. For the case of liposomes with $X_{ch} = 0$, the "black holes" are rapidly lost, at and above T_c for the PC, to give a homogenously fluorescent field as shown in 4*b*. We conclude that this indicates permeation of the bilayers by FL. "Black holes" are demonstrated by preparations with $X_{ch} = 0.5$ for more than 1 hr at room temperature following the heating period. We conclude that this indicates lack of permeation of the bilayers by FL. Initial magnification was 400× in all cases and photographic enlargement was identical. A group of particularly large liposomes was chosen for 4 (*c*) and (*d*) to show detail, but many smaller liposomes are included in which the "black hole effect" is clearly apparent. A field showing many liposomes (having diameters approximately 10 to 20 microns) was chosen for 4 (*a*) and (*b*) to show that the effect is very general

above and below T_c for the PC (41°C in this case). Thus permeability correlates with susceptibility to staining with DL (and the proposed presence of PC domains) rather than to fluidity, as measured by the rate of lateral diffusion of lipids using fluorescence recovery after photobleaching. [At $T > T_c$, the rate of lateral diffusion is decreased by $X_{ch} > 0.2$, whereas at $T < T_c$, the rate of lateral diffusion is increased by $X_{ch} > 0.2$ (Rubenstein, Smith & McConnell, 1978)].

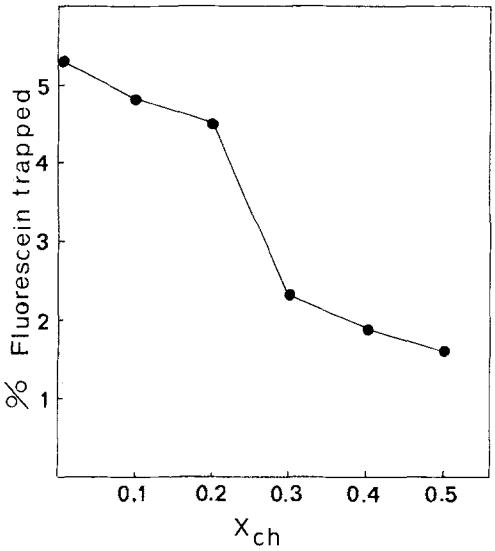

Fig. 5. Permeability of liposomes towards FL. Using the procedures described in the text, liposomes were exposed to external aqueous FL at 42°C for 1 hr. They were then cooled prior to determination of the % FL trapped relative to that originally present

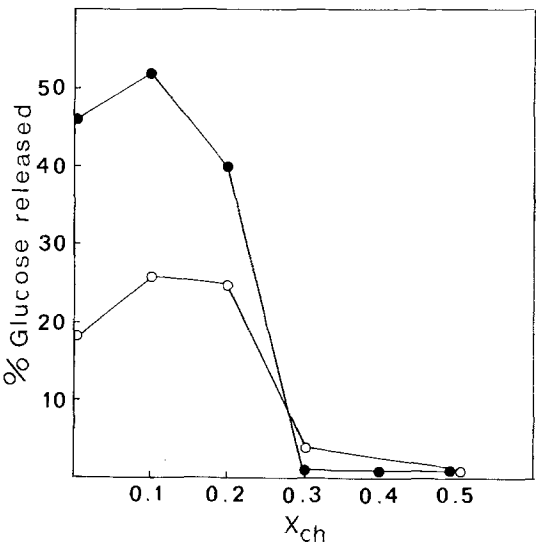

Fig. 6. Glucose release from DPPC-cholesterol liposomes as a function of X_{ch}. Liposomes were maintained at 46°C for 75 min (–●–), or 22°C for 6 hr (–○–) prior to determination of the percent of the trapped marker released as described in the text

In conclusion, the results presented here are consistent with the hypothesis that some synthetic and biological membranes are structurally different from others in that they have undergone lateral phase separation (which may or may not be ordered) to produce domains of PL; furthermore, that these PL domains are selectively stained by DL and are highly permeable to FL. We are particularly interested in the possibility that changing the permeability of a membrane, as a consequence of the aggregation of its proteins, may be a physiological mechanism for transduction of signals controlling cellular activation and/or inactivation. Cellular immune responses (e.g. those involving B cells) are frequently triggered by agents capable of aggregating (or redistributing) membrane proteins. (For a general reference *see* Braun and Unanue, 1980).

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