Interaction of Neutral Polysaccharides with Phosphatidylcholine Multilamellar Liposomes. Phase Transitions Studied by the Binding of Fluorescein-Conjugated Dextrans†

Maurizio Minetti,* Patrizia Aducci, and Vincenza Viti

ABSTRACT: The interaction of neutral polysaccharides with membrane models was studied by determining the binding of fluoresceinylthiocarbamoyl-dextran (FITC-D) to phosphatidylcholine multilamellar liposomes. The amount of FITC-D bound to liposomes increased with temperature and showed two sharp changes at temperatures around the phase transitions of synthetic phosphatidylcholine multilamellar liposomes. The temperatures midways between these abrupt changes correspond well with the temperature of prephase and phase transitions. At temperatures around the prephase transition, FITC-D adsorption isotherms suggested that the increase in bound polymer was due to an increase in the affinity of liposome membrane for FITC-D. Proton nuclear magnetic resonance data in this range of temperature showed a slight increase in the relaxation times of the phospholipid polar head group. The enhancement of the ^{31}P (^{1}H) nuclear Overhauser effect characteristic of the polar head group of phosphatidylcholine was reduced in the presence of FITC-D. These data suggested that the interaction of FITC-D with liposomes induced a slightly different mobility and packing of phospholipids which is responsible for the weakening of the association of polar head groups with the bilayer. At the gel–liquid-crystalline phase transition, fluorescence and electron microscopy data showed that the second increase in the FITC-D bound to liposomes was due to the formation of unilamellar vesicles with the polymer internalized within the vesicle. The increased disorder of the bilayer, necessary for the formation of vesicles, may also be induced by cholesterol even at low temperature.

Natural polyglycans are widely occurring constituents of biological membranes (Cook & Stoddart, 1973), and carbohydrate-containing substances seem to be good candidates for participating in cell recognition and in the binding of regulatory molecules (see review by Hughes, 1975). Little is known, however, about the structural organization and the possible interactions between polysaccharides and other constituents of the cell membrane.

One possible approach to an investigation into the arrangement of polysaccharides on the cell surface and polysaccharide involvement in biological processes is the study of the interaction between membranes and chemically defined polysaccharides. It is known that neutral polysaccharides can interact with cell membranes and induce cell aggregation (Brooks & Seaman, 1972; Brooks, 1973; Jan & Chien, 1973; Minetti et al., 1976). Cell surface interaction with neutral polymer has been used to separate cell populations (Uhlenbruck et al., 1967; Walter et al., 1972).

Recently it has been reported that membrane surface properties other than charge are involved in cell separation by partition in polymers (Walter et al., 1976; Walter & Krob, 1977). In this respect we have proposed that the interaction between neutral polysaccharides and erythrocyte membrane occurs through adsorption binding with the lipids at the level of the water interface (Minetti et al., 1978).

The ability of phospholipids to form closed lipid bilayers when suspended in an excess of aqueous solution (liposomes) as well as the degree of correspondence between the behavior of model systems and natural membranes (Kimelberg, 1976) led to their use as models in the study of the interactions between macromolecules and membrane components.

The system chosen to study the relationship between neutral polysaccharides and natural membranes has been the one constituted by fluorescent dextrans[1] (FITC-D) and multilamellar phosphatidylcholine liposomes.

The evidence reported in this paper shows that FITC-D can be adsorbed at the surface of phosphatidylcholine liposomes at temperatures at which their fatty acyl chains are in the gel–crystalline state. At temperatures around the prephase transition, the amounts of FITC-D bound to liposomes are markedly increased.

Above the main phase-transition temperature, unilamellar vesicles are formed in which FITC-D is entrapped within the vesicles. Studying the phospholipid head-group conformation by ^{1}H and ^{31}P NMR, the interaction with FITC-D is interpreted in terms of change in the packing and mobility of polar head groups.

All the observed phenomena suggest that FITC-D can be used as a useful probe in the physicochemical characterization of liposomes.

Experimental Procedure

Materials. FITC-D and dextran T fractions were obtained from Pharmacia (Uppsala, Sweden). [^{3}H]Dextran was purchased from the Radiochemical Centre (Amersham, England). Synthetic phosphatidylcholines were obtained from Calbiochem (San Diego, CA). Cholesterol was obtained from Supelco, Inc. (Bellefonte, PA).

Preparation of Multilamellar Liposomes. Multilamellar liposomes were prepared by the method of Bangham et al. (1967) by the dispersion of 10 mg of lipids in 1 mL of 0.15 M NaCl and phosphate buffer, pH 7.6, at temperatures above the phase transition. Before use, the liposomes were washed by centrifugation at 14000*g* for 5 min and resuspended in the same buffer to remove any nonmultilamellar structures present.

† From the Department of Cell Biology and Immunology, Istituto Superiore di Sanità, V. Regina Elena 299, Rome, Italy. *Received July 10, 1978.*

[1] Abbreviations used: FITC-D, fluoresceinylthiocarbamoyl-dextrans (the number following FITC-D indicates the approximate molecular weight in thousands); DMPC, DPPC, and DSPC, 1,2-dimiristoyl-, 1,2-dipalmitoyl-, and 1,2-distearoyl-3-*sn*-phosphatidylcholine, respectively; ^{1}H, ^{31}P NMR, proton and phosphorus nuclear magnetic resonance; NOE nuclear Overhauser effect; NOEE, NOE enhancement; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; DPH, 1,6-diphenyl-1,3,5-hexatriene.

0006-2960/79/0418-2541$01.00/0

Mixed cholesterol–DPPC–liposomes dispersions were obtained by dissolving the exact amount of cholesterol and DPPC in chloroform. The solvent was removed by a nitrogen stream and the mixture stored overnight under vacuum. The purity of all phosphatidylcholines was confirmed by thin-layer chromatography on silica gel G plates with the solvent chloroform–methanol–water (65:25:4). Chromatograms of all synthetic phosphatidylcholines showed only one spot. Cholesterol was determined by the standard cholesterol oxidase method.

Incubation of Polymers with Liposomes. Two methods were used to study the interaction of FITC-D with liposomes. Method a: liposome dispersions (10 mg/mL) and equal volumes of the saline–phosphate buffer containing FITC-D (the amounts of polymer are reported in the legends of the figures) were mixed at temperatures preceding the prephase transitions of phosphatidylcholines and the mixture was slowly heated (0.5 °C/min). At the temperatures indicated, heating was stopped for 20 min and aliquots of liposome–FITC-D dispersions were cooled and rapidly centrifuged at 14000*g* for 5 min. Excess FITC-D was removed by aspiration. The pelleted liposomes were suspended and washed three times with saline–phosphate buffer, pH 7.6. Method b: samples containing liposomes (10 mg/mL) and samples with FITC-D were heated separately and mixed only when the chosen temperature was reached. After 20 min of incubation, the liposome and FITC-D dispersions were cooled and centrifuged at 14000*g* for 5 min and finally processed as in method a. The exact temperature inside the liposome–FITC-D suspensions was checked by a digital thermometer equipped with a 4 × 0.5 cm probe (Contraves, Rome, Italy).

Fluorescence Microscopy and Fluorescence Determination. After the last wash, fluorescent liposomes were resuspended and observed using a Leitz Orthoplan fluorescence photomicroscope. Determination of bound FITC-D was performed by dissolving pelleted liposomes in 1.2 mL of 10% (w/v) sodium dodecyl sulfate at 50 °C. Fluorescence was measured with an Aminco-Bowmann spectrofluorometer (American Instrument Co., Silver Spring, MD). Cholesterol–DPPC mixtures were processed in the same way, except that they were dissolved in sodium deoxycholate instead of sodium dodecyl sulfate. Detergents did not modify the excitation and emission maxima of FITC-D.

Incubation with Labeled Dextran. Experiments with labeled dextran were performed with 8.5 μCi of [^{3}H]dextran (mol wt 77000; specific activity 30 mCi/g) and 2.7 mg of dextran T-70. Liposome–dextran mixtures were incubated according to method a and dissolved in the same way as FITC-D. Dissolved liposomes were mixed with Insta-gel (Packard, Downers Grove, IL) scintillation cocktail and counted with a Packard Tri-Carb liquid scintillation spectrometer.

Electron Microscopy. DPPC were examinated with a Siemens Elmiskop 102 electron microscope. For the negative contrast, DPPC–liposomes were mounted on copper grids covered with a thin carbon film and stained with sodium phosphotungstate.

For the freeze-etching technique, DPPC–liposomes were directly frozen in partially liquid Freon without any pretreatment. The specimens were cleaved in a Balzers high vacuum freeze etch unit BA 360M with a stage temperature of −100 °C, etched for 2 min, and shadowed with platinum–carbon at an angle of 45°. The vesicles were digested from the replica by Chlorox.

NMR Measurements. ^{1}H and ^{31}P NMR spectra were recorded on a Varian XL-100 instrument, operating in Fourier transform mode at 100.1 and 40.5 MHz, respectively. T_1 relaxation times were obtained employing the π–τ–(π/2) pulse sequence. Partial relaxed spectra were analyzed using a linear curve fitting. ^{31}P (^{1}H) NOEE were evaluated by comparison of intensities of fully decoupled spectra to those with the decoupling frequency gated in order to eliminate the NOE (Freeman et al., 1972). Intensities were determined by cutting and weighing the spectra.

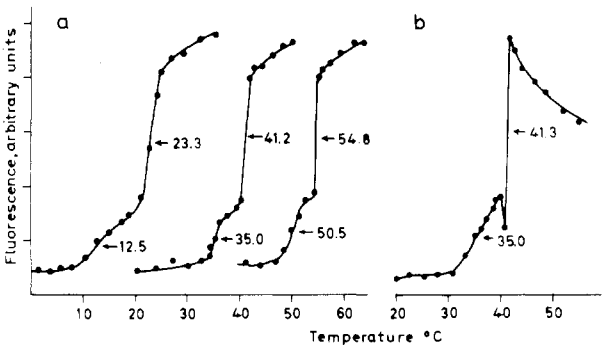

FIGURE 1: FITC-D 150 bound to DMPC–, DPPC–, and DSPC–liposomes as a function of temperature. (a) FITC-D 150 and DMPC–, DPPC–, and DSPC–liposomes (from left to right) mixed at low temperature, heated, and processed according to method a of the Experimental Procedure. (b) FITC-D 150 and DPPC–liposomes mixed after heating according to method b. Final concentrations: FITC-D 150, 3 mg/mL; phospholipids, 5 mg/mL. Fluorescence: 484-nm excitation; 525-nm emission.

Sonication of 0.15 M NaCl–phosphatidylcholine dispersions was performed under a nitrogen atmosphere with an MSE sonicator (Sussex, England) for periods of 20 s, with 1-min intervals, until the suspension was translucent. Phosphatidylcholine concentration was 40–50 mg/mL. Dextran T fractions and FITC-D 150 were lyophilized by ^2H_2O and dissolved in the lipid dispersions at a concentration of 20 mg/mL.

Turbidity Measurements. Liposomes were incubated with FITC-D 150 according to method a and washed three times with saline-phosphate buffer, pH 7.6. The pelleted liposomes were suspended in the same buffer at a final concentration of 1 mg/mL. Liposome suspensions were vigorously stirred and the turbidity change with time was monitored at 436 nm with a Cary 17 spectrophotometer. The samples, during the measurement, were kept at 25 °C.

Results

Binding of FITC-D 150 with Multilamellar Liposomes. The fluorescence of FITC-D 150 bound to DMPC–, DPPC–, and DSPC–liposomes at different temperatures is shown in Figure 1a. Two abrupt increases in the amount of FITC-D 150 bound to liposomes are visible, and the temperature midway between these abrupt changes corresponds well with the temperatures of the prephase and phase transition of multilamellar DMPC–, DPPC–, and DSPC–liposomes, as measured by differential scanning calorimetry (Hinz & Sturtevant, 1972). The different curve reported for DPPC–liposomes in panel b of Figure 1 is due to the different method used to study the binding of FITC-D to liposomes. Curves of Figure 1a were obtained by mixing liposomes and FITC-D 150 before the transition temperatures and slowly heating the mixture; for the curve of Figure 1b, FITC-D 150 and liposomes were heated separately and mixed only when the chosen temperature was reached (see methods a and b under Experimental Procedure). Figure 1 shows that the temperature midway between the increases in the FITC-D 150 bound to liposomes is identical for the two methods, but in Figure 1b

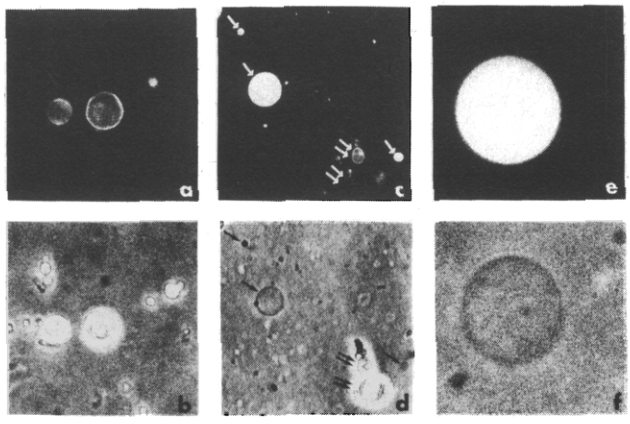

FIGURE 2: Fluorescence and phase-contrast photomicrographs of FITC-D 150 bound to DPPC–liposomes. (a–b) Liposomes after incubation at 37 °C, observed by fluorescence (a) and phase-contrast (b) microscopy, 300.8×. (c–d) Liposomes after incubation at 42 °C; the same structures were observed by fluorescence (c) and phase-contrast, 300.8×; the arrow indicates the newly formed fluorescent vesicles; double arrows indicate multilamellar liposomes with a faint external fluorescence. (e–f) Detail of vesicle observed by fluorescence and phase-contrast microscopy, 601.6×.

the amount of polymer bound to liposomes is maximal near the phase-transition temperatures and decreased thereafter. It is evident that, by using method a, the amount of FITC-D bound to liposomes for each determination is cumulative of those bound at lower temperatures. This means that the polymer bound above the transition temperatures does not revert from the liposomes at higher temperature, and thus the decrease of Figure 1b cannot be measured. We observed, in addition, that, by heating DPPC–liposome–FITC-D 150 suspensions above the prephase transition temperature (37 °C) and by subsequent cooling at room temperature, the amount of polymer bound to liposomes did not change.

To investigate the morphological aspects of multilamellar bilayers after the interaction with FITC-D 150, we observed DPPC–liposomes by fluorescence microscopy after washing out the excess polymer. At temperatures above the prephase transition (37 °C), FITC-D 150 was bound to the external surface of multilamellar liposomes. In Figures 2a and 2b, in fact, by observing the same liposome by phase-contrast and fluorescence microscopy, FITC-D 150 was present only on the external bilayer. Fluorescence was easily reduced by additional liposome washings, suggesting a weak adsorption binding. Similar adsorption patterns have been reported in the FITC-D–erythrocyte system (Minetti et al., 1978). The same localization of fluorescence was observed with liposomes treated with FITC-D 150 before the prephase transition temperature (up to 30 °C) except that the intensity of fluorescence was weaker (data not shown). On the contrary, after heating the suspension above the temperature of the gel–liquid-crystalline phase transition (42 °C), very brilliant vesicles were formed in the sample. As shown in Figures 2c and 2d, there is no correspondence between fluorescent vesicles and the characteristic multilamellar structures of DPPC–liposomes. Fluorescent vesicles appear with a peculiar circular profile (Figure 2e and 2f) and observed at phase contrast (Figure 2f) show a structure formed apparently by only one bilayer. It is to be emphasized that the newly formed vesicles represented the most fluorescent structures present in the samples, whereas the multilamellar liposomes still present showed a faint fluorescence localized at the outer monolayer not easily detectable in photographs (Figure 2c). At variance with the observation made with the low-temperature adsorption phenomena, FITC-D 150 was not released from fluorescent

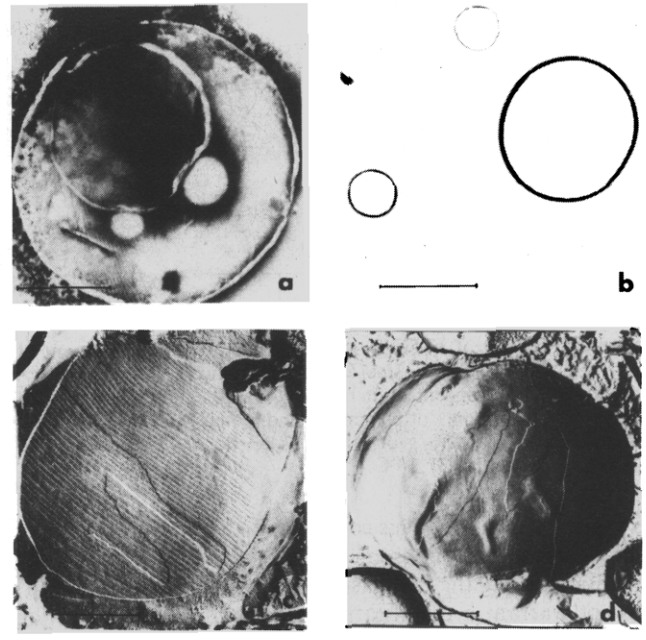

FIGURE 3: Electron microscopy of DPPC–liposomes after incubation with FITC-D 150 at 42 °C. (a) Multilamellar liposomes observed by negative contrast; (b) unilamellar vesicles seen by negative contrast; (c) fracture face of a characteristic multilamellar structure; (d) fracture face without the parallel lines observed in c attributed to the newly formed unilamellar vesicles. The small plaques detectable on the vesicle surface are generally considered either contamination artifacts or relaxation of strains in the lipid after the passage of the knife (Koehler, (1973). The bars represent 1 μm.

vesicles, thus suggesting an internalization of fluorescent polymer within the bilayer. Electron microscopy data confirmed the formation of vesicles in the samples heated above the main phase-transition temperature (42 °C) and showed that the new structures formed consisted of only one bilayer. Electron microscopy observation by negative contrast technique showed, in fact, that, in addition to the characteristic multilamellar liposomes (Figure 3a), unilamellar structures of variable size were also present (Figure 3b). Freeze-etching replicas showed faces similar to those reported by Pinto da Silva (1971) with the characteristic parallel lines of multilamellar DPPC–liposomes (Figure 3c). These multilamellar structures coexisted in the same sample with smooth fracture faces (Figure 3d) tentatively attributed to the unilamellar vesicles. Comparing data from fluorescence and electron microscopy, the intensely fluorescent vesicles could be conceivably ascribed to the unilamellar vesicles observed by electron microscopy. Small unilamellar vesicles with an estimated size of 150–300 Å can, however, be obtained from multilamellar liposomes after prolonged sonication (Tyrrell et al., 1976). The unilamellar structures formed after FITC-D 150 interaction in our system were larger than those obtained by sonication and reached a maximum size of 20–30 μm.

Dependence of FITC-D Concentration and FITC-D Molecular Weight. The dependence of FITC-D 150 concentration on the binding of polymer on DPPC–liposomes at temperatures before and after the prephase transition is given in Figure 4 as a log–log plot. At these temperatures, the FITC-D binding was localized only on the external surface of liposomes (Figure 2a) and both the morphology and the amount of FITC-D bound to liposomes were independent of the incubation time. It is, however, to be strengthened that the adsorption isotherms of Figure 4 were obtained after three washes of the samples and, therefore, represent an underestimation of the polymer actually bound to the liposomes when an excess of FITC-D

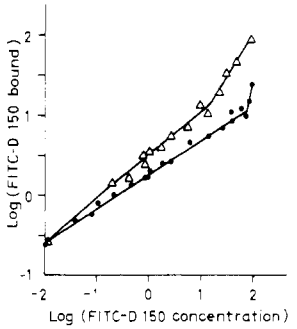

FIGURE 4: Effect of FITC-D 150 concentration on the amount of polymer bound to DPPC–liposomes. Incubation was performed as in Figure 1a; the concentration of FITC-D 150 added was expressed as mg/mL; the amount of polymer bound to liposomes was expressed as mg of FITC-D 150/g of DPPC. (●—●) Isotherm at 25 °C; (△—△) isotherm at 37.2 °C.

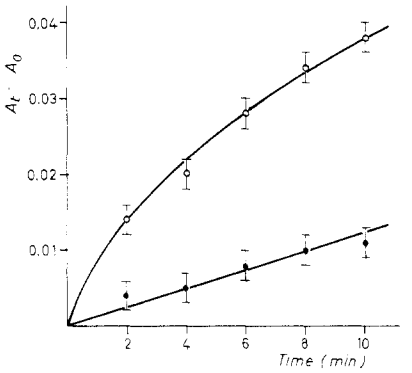

FIGURE 5: Turbidity changes at 436 nm of DPPC–liposomes preincubated with FITC-D 150 at 37.2 °C. The absorbance of a sample containing liposomes preincubated with FITC-D 150 and washed three times was compared with that of a reference containing only liposomes. The FITC-D 150 concentration used during the preincubation was 30 mg/mL (open symbol) and 3 mg/mL (closed symbol). A_0 and A_t are the absorbance of the dispersion at time $t = 0$ and t.

is present in solution. This consideration and the heterogeneity of the adsorbent (liposomes with sizes and shapes likely modified during the prephase transition, Janiak et al., 1976) do not warrant a quantitative treatment of the adsorption isotherm. Nevertheless, our isotherm fit the empirical equation of Freundlich, which seems to work satisfactorily with the adsorption of solutes by solids (Prutton & Maron, 1956). The reported isotherms, however, are useful for comparing the differences in the behavior of the liposome–FITC-D system around the prephase transition temperatures. As shown in Figure 4, the isotherms show a statistically significant difference of the slopes (the analysis of variance for differences between regression slopes give a significant test F value, $p < 0.05$). Considering the method used to detect bound FITC-D 150, also a difference in the rate of polymer release from the liposome surface before and after the prephase transition could account for the observed difference in the slope. A reduced rate of release could be the result of an increased affinity of the liposome surface for the polymer after the prephase transition. The isotherms of Figure 4 do not show any indication of saturation, but the slopes show a marked increase at high polymer concentration. Figure 4, in fact, shows that, at FITC-D 150 concentrations of 55 mg/mL and of 13.2 mg/mL, there is a marked increase in the slope of the isotherm at 25.0 and 37.2 °C, respectively. At high FITC-D 150 concentration, we observed a formation of stable aggregates of multilamellar liposomes also after washing out the excess of fluorescent polymer. For these samples, the increased formation of aggregates was suggested also by the increase in the absorbance at 436 nm with time. Figure 5, in fact, shows that the aggregation of liposomes preincubated with FITC-D 150 at 37.2 °C and washed three times is more pronounced at high polymer concentration. Liposome aggregation could induce a trapping of polymer in the aggregates with a resulting increase in the amount of polymer associated with liposomes. In this respect, it is interesting to observe that the increase in the slope of the 25.0 and 37.2 °C isotherms occurs when a similar amount of FITC-D 150 is bound at the liposome surface (about 12 mg/mL). However, it should be considered that other phenomena, such as a polymer penetration into the outer leaflets of the liposomes, without unilamellar vesicle formation, could also account for both the difference in the slope of the two isotherms and for the increase in the slopes at high polymer concentration. In this latter hypothesis, FITC-D-liposome interaction could be conveniently described as sorption phenomena.

At 41.9 °C, when the concentration of FITC-D 150 exceeded 0.32 mg/mL, the phenomenon of unilamellar vesicle formation was evident and the amount of FITC-D 150 bound to liposomes markedly increased. By raising the concentration of polymer in the liposome suspension the number of fluorescent vesicles rapidly increased and reached an apparent saturation at a very high polymer concentration. The maximum uptake of FITD-D 150 obtained by us was about 178 mg/g of DPPC.

Table I: Dextran Bound on DPPC Liposomes at Different Temperatures; FITC-D Molecular Weight Dependence

dextran	mol wt[a]	mg of bound polymer[b]/g of DPPC 25.1 °C	37.2 °C	41.9 °C
FITC-D 3	2820	0.2	0.9	2.0
FITC-D 20	19000	0.3	1.5	7.5
FITC-D 40	39000	0.4	1.8	11.2
FITC-D 70	68500	0.4	1.7	9.0
FITC-D 150	154000	3.5	7.0	25.0
[^{3}H] dextran[c]	70000	0.8	1.1	1.8

[a] Dextran molecular weights are manufacturer's data. [b] Incubation was performed according to method a at a final FITC-D concentration of 3 mg/mL. The accuracy of the measurement is about ±0.1 mg/g of DPPC. [c] Mixture of [^{3}H] dextran (mol wt 77 000) and dextran T 70 (mol wt 70 000).

The amount of FITC-D bound to DPPC–liposomes strongly depends upon the polymer molecular weight. Table I reports the amounts of bound FITC-D/g of DPPC for polymers with molecular weights ranging from 2820 to 154000 at three characteristic temperatures (25.1, 37.2, and 41.9 °C). It is evident that both the surface phenomena and the formation of unilamellar vesicles depends upon the polymer molecular weight. The evidence that stable FITD-D binding can be obtained with high molecular weight polymer suggests a multipoint binding mechanism between polymer chain and liposome surface. Stable interaction seems to be necessary also to induce the formation of unilamellar vesicles when the phospholipid is heated above the main phase-transition temperature. Table I, in fact, shows that with FITC-D 3 the polymer uptake, although present, was about 12 times smaller than with FITC-D 150.

In order to determine whether FITC–lipid interaction is responsible for the binding of FITC-D to liposomes, control experiments were run with FITC alone and ^{3}H-labeled dextran. FITC alone penetrates rapidly in the liposomes independently of their gel- or liquid-crystalline state and does not induce any

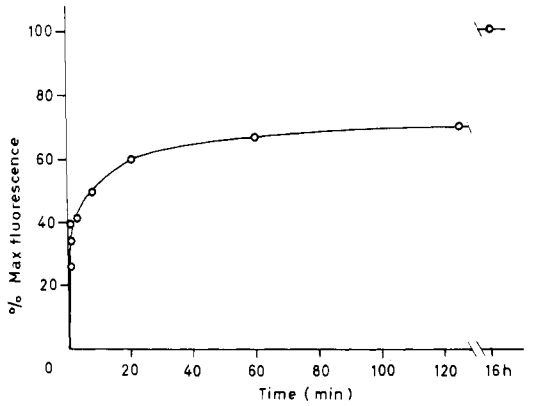

FIGURE 6: Rate of vesicle formation in FITC-D 150-liposome interaction at 42 °C. Polymer and liposome suspension were heated separately at 42 °C and mixed, avoiding the cooling of sample. At the indicated times, aliquots of 0.3 mL were taken up and processed as indicated under Experimental Procedure. Polymer and liposome concentration were as in Figure 1. Bound fluorescence was expressed as a percentage of the maximum fluorescence obtained after 16 h of incubation.

vesicles formation by heating liposomes above the main phase-transition temperature. As shown in Table I, the neutral polysaccharides of 70000 molecular weight and FITC-D 70, at the indicated concentrations, are adsorbed to a similar extent below the main phase-transition temperature. Moreover, experiments with [^{3}H]dextran 70 at 41.9 °C do not show the abrupt increase in the amount of polymer bound to liposomes (Table I). However, if the polysaccharide is modified with FITC, the resulting molecule stimulates the formation of unilamellar vesicles. This characteristic seems to be absent in [^{3}H]dextran, at least for the molecular weight investigated.

Rate of Vesicle Formation in FITC-D 150–Liposome Interaction. The formation of unilamellar vesicles, that takes place when FITC-D 150 and DPPC–liposomes are heated above the main phase-transition temperature, showed the kinetics reported in Figure 5. Expressed as a percentage of the total bound fluorescence, it is evident that the 50% value was reached in a few minutes (i.e., 8 min), while the remaining 50% was reached only after 16 h. The binding of FITC-D 150 to DPPC–liposomes, thus, seems to be the result of at least two phenomena: the first, accomplished in a few minutes, that accounts for about half of the total fluorescence, and the second, very strongly dependent on time. Multilamellar liposomes and vesicles present in the sample were observed by fluorescence microscopy at various times. After a few minutes and up to 30 min, the fluorescence pattern was as reported in Figure 2: many fluorescent unilamellar vesicles and multilamellar liposomes with only a faint external fluorescence. With increasing time, the pattern slowly changed, and at the end of incubation (16 h) unilamellar vesicles were remarkably reduced, while many multilamellar liposomes appeared intensely fluorescent with a fluorescence localized also in the internal bilayers. In fact, Figure 6 shows multilamellar fluorescent liposomes and a unilamellar vesicle coexisting in the sample with about the same fluorescence intensity.

Effect of Cholesterol on FITC-D–Liposome Interaction. The interaction between cholesterol and phospholipid has been intensively investigated by a variety of physical techniques. Cholesterol has a condensing effect, reduces the chain mobility of phospholipids in the liquid-crystalline state, and changes the sharp cooperative gel–liquid-crystal transition to a diffuse noncooperative event. On the other hand, cholesterol increases the chain mobility of phospholipids in the gel–crystalline state, the so-called liquefying effect (see review by Lee, 1975).

Table II: Effect of Cholesterol on FITC-D 150 Bound to Liposomes[a]

cholesterol:DPPC molar ratio	mg of bound FITC-D 150/g of DPPC		
	0.2 °C	37.2 °C	42.0 °C
0:1	1.2	3.0	9.5
0.5:1	2.7	2.7	4.9
1:1	6.2	8.4	11.2
2:1	13.2	22.6	29.4

[a] Incubation was performed according to method a described under Materials and Methods at a final FITC-D 150 concentration of 1 mg/mL.

To obtain information about the role of bilayer fluidity in the interaction of FITC-D with DPPC-liposomes, we carried out some preliminary experiments with liposomes containing increasing quantities of cholesterol. In the presence of a cholesterol:DPPC molar ratio of 0.5:1, the amount of FITC-D 150 bound to liposomes did not show the characteristic change after the prephase transition since cholesterol increased the amount of polymer bound at low temperature (Table II). In addition, with this amount of cholesterol, the second increase due to the formation of unilamellar vesicles was significantly reduced (Table II). With a 1:1 molar ratio of cholesterol to DPPC, the addition of FITC-D 150 induced, already at 0.2 °C, the formation of fluorescent unilamellar vesicles, and a pronounced binding of the polymer (Table II). With increasing temperatures, both the number of fluorescent vesicles and the bound FITC-D 150/g of lipid mixture increased (Table II). At high temperature, the unilamellar vesicles were larger in size than DPPC–liposomes but morphologically similar to the structure shown in Figure 2. Recently, it has been reported that, by using an initial excess of cholesterol, it is possible to obtain stable liposome preparations with 2:1 molar ratio with DPPC. Therefore, in accordance with Lundeberg (1977), we used an initial molar ratio of cholesterol of 2.5:1 to obtain a stable 2:1 multilamellar preparation. At 0.2 °C, these liposomes showed more bound FITC-D 150/g of lipid than DPPC–liposomes after the main phase-transition temperature (Table II). Also in this case the bound fluorescence was due to a massive formation of large unilamellar vesicles. Data in Table II show in addition that incubation at increasing temperatures resulted in an even higher amount of polymer bound to liposomes.

The effect of cholesterol on the binding of FITC-D on liposomes was also a phenomenon depending upon the polymer molecular weight. At 0.2 °C, for example, the amount of FITC-D 3 bound to 2:1 cholesterol–DPPC–liposomes was about nine times lower than with FITC-D 150.

NMR Studies of Dextran–Liposome Interaction. ^{1}H and ^{31}P NMR spectra of sonicated liposomes have been examined in the presence and in the absence of dextrans before and after the gel–liquid-crystalline phase transition. Before this transition, the proton $-(CH_2)_n$ resonances and the signal due to the terminal methyl group are barely detectable. We have, therefore, examined the behavior of the choline methyl groups and in particular the proton spin–lattice relaxation time T_1. As we can see in Table III, a weak (approximately 20%) but reproducible increase of T_1 in the presence of dextrans T 150 and T 2000 and FITC-D 150 has been observed for DPPC– and DMPC–liposomes at temperatures below their respective phase transitions. The increase of T_1 in vesicle–dextran suspensions was statistically significant. In fact, the analysis of variance for differences between regression slopes obtained for vesicles with and without dextrans gave a high significant

Table III: Dextran Effects on the NMR Parameters of the Polar Head Group

lipid	substance tested	temp (°C)	$-N(CH_3)_3^+$ T_1 (s)	$^{31}P(^1H)$ NOEE[a] (%)
DMPC	none	18 ± 1	0.21 ± 0.01	30 ± 2
	FITC-D 150	18 ± 1	0.26 ± 0.01	21 ± 1
	dextran T 2000[b]	18 ± 1	0.25 ± 0.01	18 ± 1
	none	35 ± 1	0.33 ± 0.01	41 ± 2
	FITC-D 150	35 ± 1	0.34 ± 0.01	32 ± 2
	dextran T 2000	35 ± 1		29 ± 2
DPPC	none	34 ± 1	0.27 ± 0.01	37 ± 2
	FITC-D 150	34 ± 1	0.41 ± 0.01	24 ± 1
	dextran T 150[c]	34 ± 1	0.38 ± 0.01	29 ± 2
	dextran T 2000	34 ± 1	0.37 ± 0.01	28 ± 2
	none	54 ± 1	0.56 ± 0.01	64 ± 3
	FITC-D 150	57 ± 1		50 ± 3
	dextran T 2000	57 ± 1	0.56 ± 0.01	51 ± 3

[a] NOEE is reported as % enhancement of signal intensity. [b] Molecular weight about 2 000 000. [c] Molecular weight 154 000.

test F value, $p < 0.01$. At temperatures above the main phase transition of phospholipids, we could not observe any difference of ^1H spin–lattice relaxation times in the absence or in the presence of dextran T 2000 or FITC-D 150 (Table III).

The involvement of the polar head group in the interaction with dextran is further demonstrated by the decrease in the ^{31}P (^1H) NOEE observable in the presence of polysaccharides. Recently ^{31}P NMR has been demonstrated to be a useful probe of the head-group region. In particular ^{31}P (^1H) NOE has been used to determine the conformation of the polar head group in natural and synthetic phospholipids (Yeagle et al., 1975–1977). The NOE arises from protons interacting in dipolar fashion with the phosphorus, and generally the protons must be close to the phosphorus to cause the effect (Yeagle et al., 1975). The maximum effect on the NOE arises from the *N*-methyl protons of neighboring phospholipids (Yeagle et al., 1975–1977). In Table III we can observe both for DMPC and for DPPC a decrease of the NOEE in the presence of FITC-D 150 or of dextans T 150 and T 2000. The effect is more pronounced before the gel–liquid phase transition and slightly weaker for DPPC than for DMPC (Table III). In the case of DSPC, both the variations of the ^1H relaxation times and of ^{31}P (^1H) NOEE are small (i.e., T_1) or undetectable (i.e., NOEE). This could be related to the different behavior of ^{31}P NMR spectra of DSPC with respect to DMPC and DPPC observed in multilamellar liposomes by Cullis et al. (1976). The authors interpreted the difference in terms of increased motion of DSPC polar head group well below the phase-transition temperature. Low molecular weight dextran T 10 has no effect on both the relaxation times of the polar heads and on the NOEE (data not shown). It is known (Lentz et al., 1976) that the gel–liquid transition temperatures for sonicated vesicles is lower and less well defined than for multilamellar liposomes. For this reason, temperatures at which NMR spectra are measured are just before and after the main phase transition of phospholipid vesicles as can be determined by ^{31}P NMR spectra (McLaughlin et al. 1975; V. Viti and M. Minetti manuscript in preparation).

Discussion

The data reported in this paper show that the interaction of FITC-D with multilamellar liposomes depends upon both the mobile state of lipid fatty acid chains and the mobility and packing of polar head groups.

It should be pointed out that the phase-transition temperatures of phosphatidylcholines measured with FITC-D were always reproducible and, especially for the gel–liquid crystalline phase transition, the temperature ranges of the transition lay within a few degrees (Figure 1). On the other hand, the quantitative aspects of this technique were not equally satisfactory. The amounts of polymer bound to liposomes, in fact, were reproducible only with the same preparation of liposomes. On the contrary, on using different liposome preparations, the amount of bound FITC-D 150/g of DPPC differed by about ±30%. This variability depends upon the condition used for the formation of multilamellar liposomes (i.e., heating above the phase-transition temperature and 2–3 h of swelling). The swelling conditions, in fact, are important in determining the proportion of exposed lipid (Scwartz & McConnell, 1978) and, thus, the surface disposable for FITC-D. On using liposome preparations stored for at least 24 h at 4 °C, we observed that the amount of bound FITC-D 150/g of DPPC changed, between the different preparations, by only ±7%. Long hydration times, in fact, favor the formation of liposomes with a constant proportion of external lipid (Schwartz & McConnell, 1978). The presence in the liposome preparation of structures with different reactivity for FITC-D was suggested also by the pattern of the kinetic experiment reported in Figure 5. The apparent rapid saturation of the vesicle formation phenomenon suggests that in the liposome preparation there exist two liposome populations: one that can rapidly form internally fluorescent vesicles and the other that in the first 10–20 min shows only a faint external fluorescence. Otherwise, one would have expected a complete formation of vesicles from multilamellar liposomes in a few minutes, without the slow kinetic observed (Figure 6). In this respect, it is possible to explain the poor reproducibility observed between different preparations, by assuming a variability in the ratio between the two proposed liposome populations.

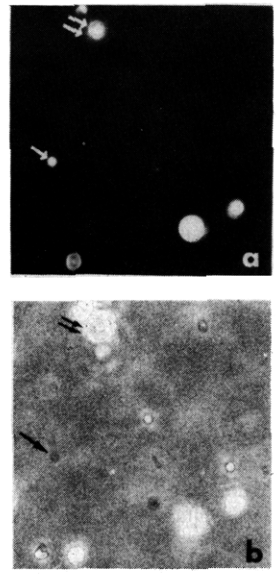

FIGURE 7: Effect of long incubation times at 42 °C on the fluorescence pattern of liposomes. Samples of Figure 5 after 16 h of incubation observed by fluorescence (a) and phase-contrast (b) microscopy, 414×; experimental conditions were as in Figure 5. The arrow indicates an unilamellar vesicle; note the correspondence between the fluorescence and the phase-contrast patterns and the internalization of fluorescence evident in the liposome on the top left (double arrows).

The newly formed vesicles were unstable with time and after several hours multilamellar liposomes, showing internal fluorescence, were observed (Figure 7). A likely explanation of such polymorphism in the structure of liposomes is that, after mixing FITC-D and phosphatidylcholine liposomes above the phase-transition temperature, the adsorbed polymer may

penetrate the outer liposome bilayer with a consequent formation of vesicles. Subsequently, during the time course of the reaction at 42 °C, it can be conceived that unilamellar vesicles and multilamellar liposomes can, by a slow process, fuse into multilamellar liposomes showing internal fluorescence (Figure 7).

The increase in FITC-D bound to the multilamellar liposomes after heating the sample above the prephase-transition temperature is an interesting finding. The possibility that, after the prephase-transition temperature, there is an increase in the penetration of FITC-D into the external bilayer of liposome is to be considered. Lee (1977), in fact, suggested that premelting phenomena in bilayer could be due to a formation of "defects" or vacant sites in the packed lattice. Our data, however, seem to exclude a massive penetration of FITC-D into the external bilayer before the main phase transition since no fluorescent unilamellar vesicles were observed at these temperatures; but a reduced polymer penetration undetectable by fluorescence microscopy cannot be completely ruled out. Polymer internalization even if present does not perturb the bilayer packing and, in fact, the prephase transition and the phase-transition temperatures obtained by turbidity measurements of liposome dispersions in the presence or in the absence of FITC-D 150 were coincident and, therefore, are not shown. On the other hand, the low-temperature transition can be detected with: (i) probes located in the hydrophobic portion of the bilayer (Shimshick & McConnell, 1973; Rand et al., 1975; Lentz et al., 1976); (ii) studying optical properties of liposome suspensions (Petersen et al., 1975); (iii) X-ray diffraction studies (Janiak et al., 1976); (iv) dilatometry (Nagle, 1973) and differential scanning calorimetry (Ladbrooke & Chapman, 1969; Hinz & Sturtevant, 1972). These studies indicate a delicate balance is maintained between the hydrophobic and hydrophilic regions of the phospholipid and strongly suggest that the prephase transition involves structural changes affecting the entire molecule. The increase in the FITC-D bound to liposomes after the prephase transition suggests that the events accompanying this transition seem to favor FITC-D binding. Noteworthy, our NMR studies on sonicated liposomes show that the FITC-D interaction involves the polar head groups and was more evident below the gel–liquid-crystalline phase transition (prephase transition is undetectable in sonicated liposomes, Lentz et al., 1976). Therefore, extrapolating NMR data to the multilamellar system, prephase transition could be associated with a change in the packing and mobility of polar head groups that induce a liposome structure more suitable for FITC-D interaction. In this respect our data support the view that the prephase transition is associated with a change involving also the polar portion of DPPC. The involvement of polar head group in FITC-D–liposome interaction is demonstrated by the increase in the ^1H relaxation time of the $-N(CH_3)_3^+$ group and in the decrease of the NOEE. As shown by Yeagle et al. (1975–1977), NOE is due to the association occurring between the $-N(CH_3)_3^+$ groups and the negatively charged phosphates of neighboring lipids. Both the dextran effects indicate that polymers induce a reduction of the surface dipolar association of phosphocholine groups. This effect could be obtained with a slight unfolding of polar head groups (only small changes in the polar head-group torsion angles account for the observed effects) or with an increase in the separation between neighboring head groups caused by FITC-D. In this latter hypothesis, FITC-D could act as a "spacer" molecule between DPPC molecules. For cholesterol, a molecule known to reduce the NOEE (Yeagle et al., 1975), a spacer mechanism has been proposed to explain the effect on polar regions of DPPC (Brown & Seelig, 1978). Although FITC group, for simple hydrophobic considerations, could be spacer molecules for DPPC bilayer, the observation that similar effects were obtained with unlabeled dextrans (Table III) led us to believe the unfolding hypothesis more suitable. Further investigation is necessary to clarify the degree of polymer penetration into DPPC bilayers.

Our interpretation of dextran effects assumes that the phosphocholine head groups are oriented almost parallel to the liposome membrane both before and after phase transition. Evidence reported by Büldt et al. (1978), in fact, demonstrates that the orientation of phosphocholine polar head group before and after the two phase transitions of DPPC is parallel to the bilayer surface. This conformation should not be considered a static situation, but a preferred conformation of head group undergoing considerable motion (Gally et al., 1975).

Transition temperatures obtained with FITC-D binding correspond well with those measured by differential scanning calorimetry (Hinz & Sturtevant, 1972), suggesting that the interaction of FITC-D with liposomes does not induce a strong modification in the packing of the bilayer. This observation is of some interest mainly for the prephase transition temperature, considering that discrepancies exist between the values obtained with different physical techniques (Lentz et al., 1976). It is interesting to observe that, with hydrophobic (as DPH fluorescence depolarization) and FITC-D probes, when the chain length of the phospholipid increases, the temperature range of the main phase transition decreases (Figure 1a). This observation, interpreted in terms of increased cooperativity of the transition (Lentz et al., 1976), leads to the hypothesis that the phenomenon of vesicle formation is strictly related to the cooperativity of the phase transition.

The formation of unilamellar vesicles from multilamellar liposomes was maximum at the temperature of the gel–liquid-crystalline phase transition. As reported in Figure 1b, the formation of vesicles above the phase transition occurs, but to a minor extent. Optima for other processes have observed at the onset temperatures of lateral phospholipid phase transition (Van der Bosch & McConnell, 1975; Papahadjopoulos et al., 1973). Moreover enhanced permeability of Tempo-choline spin label at the temperature of phase transition has been demonstrated by Marsh et al (1976) in single-bilayer vesicles of DMPC. These authors, since the Tempo-choline permeability is much smaller, both above and below the transition, attributed the enhanced permeability to the "areas of mismatch in molecular packing which occurs at the interfacial regions between fluid and ordered lipids". It is possible to apply this interpretation to the phenomenon of vesicle formation induced by FITC-D. The polymer, externally adsorbed on liposomes, finds at the temperature of phase transition coexisting regions of order and of disorder in the plane of the bilayer, and the boundaries between discrete gel- and liquid-crystalline domains can be taken up by water and by FITC-D. This phenomenon can produce the fracture of the first bilayer with a formation of unilamellar vesicles with the polymer inside the membrane. Above the phase-transition temperature where the packing of liposomes must be "looser" than in the gel-crystalline phase, the unilamellar vesicles can be formed, although to a minor extent (Figure 1b).

Our interpretation of FITC-D-induced phenomena was strengthened by the experiments with mixed cholesterol–DPPC–liposomes. It has been reported that cholesterol in mixed phosphatidylcholine bilayers affects the cooperativity of phase transition and removes the prephase transition

(Ladbrooke & Chapman, 1969). In our system at 0.5:1 molar ratio, cholesterol causes an increase of the amount of polymer bound at low temperature, a disappearance of the prephase transition, and a lowering of the amount of polymer bound at the main phase-transition temperature (Table II). All these effects may be interpreted as perturbing effects in both the hydrophilic and hydrophobic portion of the bilayer. On the other hand, the disordering effect of cholesterol was unable to induce the formation of vesicles before the main phase-transition temperature if a molar excess of DPPC is present in the liposome. A clear formation of vesicles appears, in fact, only when the main phase transition temperature is reached. On increasing the amount of cholesterol, the presence of large disordered domains and the increased bilayer fluidity cause the polymer-induced formation of vesicles also at very low temperature (Table II).

Data obtained with [^{3}H]dextran clearly show that the presence of fluorescein was essential at least for the vesicle formation phenomenon. However, it should be pointed out that: (a) both the vesicle formation and the adsorption phenomenona induced by FITC-D depend upon the molecular weight of polymer (Table I), ruling out the possibility that our results are due to trapping of polymer in the pellet of liposomes or to a polymer diffusion inside spontaneously formed vesicles; (b) no similar effect could be detectable with only FITC; (c) the surface adsorption of FITC-D and dextran T fractions induces similar modifications in the organization of polar head group of phosphatidylcholines (increasing of relaxation times of polar group and decreasing of ^{31}P (^{1}H) NOEE). Taken together, the data reported suggest that the polysaccharide moiety was important for the adsorption phenomena and for the interfacial effects, while the presence of FITC groups was important for the penetration of the polymer and for the formation of vesicles.

From the cell-surface organization point of view, the data reported in the present paper suggest that natural polysaccharides "anchored" to the cell membrane (by adsorption, ionic or covalent bonds) may interact with phospholipid domains of the cell surface. In this hypothesis, it is also likely that natural polyglycans could be preferentially adsorbed in domains with a characteristic degree of packing and mobility. It is interesting to observe that recent studies (Lee, 1977; Marsh et al., 1976) suggest that some physiologically important properties of biological membranes may derive from the lipid lateral phase separation in the bilayer accompanying phase transitions.

Acknowledgments

We are indebted to F. Rosati-Valente for assistance and helpful discussion of electron microscopy results. We are grateful to E. Chessa for the excellent photographic work. We thank also Dr. M. Grandolfo for the help in statistical analysis.

References

Bangam, A. D., De Gier, J., & Greville, G. D. (1967) *Chem. Phys. Lipids 1*, 225.

Brooks, D. E. (1973) *J. Colloid Interface Sci. 43*, 700.

Brooks, D. E., & Seaman, G. V. F. (1972) *Nature (London), New Biol. 238*, 251.

Brown, M. F., & Seeling, J. (1978) *Biochemistry 17*, 381.

Büldt, G., Gally, H. U., Seeling, A., Seeling, J., & Zacchai, G. (1978) *Nature (London) 271*, 182.

Cullis, P. R., De Kruyff, B., & Richards, R. E. (1976) *Biochim. Biophys. Acta 426*, 433.

Cook, G. M. W., & Stoddart, R. W. (1973) in *Surface Carbohydrates of the Eukaryotic Cell*, p 56, Academic Press, New York.

Freeman, R., Hill, H., & Kaptein, R. (1972) *J. Magn. Reson. 7*, 327.

Gally, H. U., Neiderberger, W., & Seeling, J. (1975) *Biochemistry 14*, 3647.

Hinz, H. J., & Sturtevant, J. M. (1972) *J. Biol. Chem. 247*, 6071.

Hughes, R. C. (1975) *Essays Biochem. 11*, 1.

Jan, K.-M., & Chien, S. (1973) *J. Gen. Physiol. 61*, 638.

Janiak, M. J., Small, D. M., & Shipley, G. G. (1976) *Biochemistry 15*, 4575.

Kimelberg, H. K. (1976) *Mol. Cell. Biochem. 10*, 171.

Koehler, J. K. (1973) *Advanced Techniques in Biological Electron Microscopy*, p 90, Springer Verlag, Berlin.

Ladbrooke, B. D., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 304.

Lee, A. G. (1975) *Prog. Biophys. Mol. Biol. 29*, 3.

Lee, A. G. (1977) *Biochim. Biophys. Acta 472*, 237.

Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4521.

Lundeberg, B. (1977) *Chem. Phys. Lipids 18*, 212.

Marsh, D., Watts, A., & Knowles, P. F. (1976) *Biochemistry 15*, 3570.

McLaughlin, A. C., Cullis, P. R., Berden, J. A., & Richards, R. E. (1975) *J. Magn. Reson. 20*, 146.

Minetti, M., Aducci, P., & Teichner, A. (1976) *Biochim. Biophys. Acta 437*, 505.

Minetti, M., Teichner, A., & Aducci, P. (1978) *Biochem. Biophys. Res. Commun. 80*, 46.

Nagle, J. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3443.

Papahadjopoulos, D., Jacobson, K., Nir, S., & Isac, T. (1973) *Biochim. Biophys. Acta 311*, 330.

Petersen, N. O., Kroon, P. A., Kainosho, M., & Chan, S. I. (1975) *Chem. Phys. Lipids 14*, 343.

Pinto da Silva, P. (1971) *J. Microsc. (Paris) 12*, 185.

Prutton, C. F., & Maron, S. H. (1956) *Fundamental Principles of Physical Chemistry*, p 230, Macmillan, New York.

Rand, P. R., Chapman, D., & Larsson, K. (1975) *Biophys. J. 15*, 1117.

Schwartz, M. A., & McConnell, H. M. (1978) *Biochemistry 17*, 837.

Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2351.

Tyrrell, D. A., Heath, T. D., Colley, C. M., & Ryman, B. E. (1976) *Biochim. Biophys. Acta 457*, 259.

Uhlenbruck, G., Seaman, G. V. F., & Coombs, R. R. A. (1967) *Vox Sang. 12*, 420.

Van der Bosch, J., & McConnell, H. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4409.

Walter, H., & Krob, E. J. (1977) *FEBS Lett. 78*, 105.

Walter, H., Tung, R., Jackson, L. J., & Seaman, G. V. F. (1972) *Biochem. Biophys. Res. Commun. 48*, 565.

Walter, H., Krob, E. J., & Brooks, D. E. (1976) *Biochemistry 15*, 2959.

Yeagle, P. L., Hutton, W. C.-H., & Martin, R. B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3477.

Yeagle, P. L., Hutton, W. C., Huang, C.-H., & Martin, R. B. (1976) *Biochemistry 15*, 2121.

Yeagle, P. L., Hutton, W. C., Huang, C.-H., & Martin, R. B. (1977) *Biochemistry 16*, 4344.