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Glycophorin in Lipid Bilayers

(lipid–protein interactions/freeze-fracture electron microscopy/spin labels/glycoproteins/lateral phase separations)

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ABSTRACT **Glycophorin, the major glycoprotein of human erythrocytes, has been isolated and reincorporated into lipid vesicles. Freeze-fracture electron microscopy shows the reincorporated glycophorin to occur as small particles in vesicle fracture faces while the etch faces are smooth. The glycoprotein has a tendency to cluster into groups of several particles. Evidence is presented that, although lipids in immediate contact with glycophorin are likely somewhat immobilized, the entire lipid–protein complex has a tendency to occupy fluid regions of the bilayer. Reincorporated glycophorin assumes its proposed conformation in the intact erythrocyte in so far as it penetrates the hydrophobic membrane interior while its N-terminal end with attached carbohydrate residues is exposed to the aqueous compartment and is available as a specific recognition site.**

Human erythrocytes possess surface receptors capable of recognizing and binding a variety of external agents. Glycophorin, the major glycoprotein of human erythrocytes, carries specificity for certain plant lectins; MN [and perhaps (1) AB] blood-group substances; and influenza virus (2, 3). Glycophorin has been isolated and characterized as having a molecular weight in the neighborhood of 50,000 and being some 60% carbohydrate and 40% protein (2, 3). In the intact erythrocyte it is thought to penetrate the membrane from side to side, with its C-terminal end exposed at the inner surface (4) and a lengthy portion of its N-terminal end, bearing the majority of the carbohydrate, exposed at the outer surface (2, 3). The protein backbone in the interior of the membrane is rich in hydrophobic amino-acid residues (5). Glycophorin's function in the human erythrocyte is not known but it appears to be associated with the 85 Å membrane particles seen by freeze-fracture electron microscopy (6–8) and to be connected in some way to the spectrin network at the inner surface (8–10). We report here the successful incorporation of this glycoprotein into bilayer lipid vesicles along with the results of freeze-fracture electron microscopy and spin label studies of the systems so formed.

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC), and phosphatidylserine (from bovine brain) were obtained from Calbiochem and used without further purification. Dielaidoylphosphatidylcholine (DEPC) was synthesized according to the method of Cubero Robles and Van den Berg (11) by Dr. S. Wu in this laboratory. Egg lecithin was isolated from egg yolks according to the method of Singleton *et al.* (12) and further purified by silica gel chromatography on Silic AR CC-4, 100–200 mesh. The detergent, dodecyltrimethylammonium bromide, was prepared by Dr. B. Gaffney in this laboratory by reaction of dodecyl bromide with trimethylamine followed by recrystallization from acetone–methanol.

Samples for freeze-fracture electron microscopy were equilibrated at the temperature of interest in a water-saturated atmosphere on 3-mm copper planchets before being rapidly quenched in Freon 22 cooled in liquid nitrogen. The freeze-fracture process was carried out at −119° (−106° if the sample was to be etched) on a Balzers BAF 301 instrument. Replicas were cleaned in commercial bleach or in ethanol and examined with a Phillips EM200.

Human blood was obtained from adult donors. Cells were washed three times in phosphate-buffered saline (pH 7.4) prior to preparation of ghosts by the procedure of Dodge *et al.* (13). Glycophorin was extracted from the freeze-dried ghost membranes using 0.3 M lithium diiodosalicylate and purified according to the procedure laid out by Marchesi and Andrews (14). It was pure as judged by sodium dodecyl sulfate-gel electrophoresis on 5 and 10% polyacrylamide gels (15) and was identical to a sample kindly supplied by Dr. V. T. Marchesi.

Lipid vesicles containing glycophorin were prepared in a manner exactly analogous to that used by Hong and Hubbell (16) to incorporate rhodopsin into lipid bilayers. Lipid and glycoprotein were dissolved in 10 mM phosphate buffer containing 100–300 mM dodecyltrimethylammonium bromide and left at 4° for a minimum of 5 hr. In order to make vesicles comprised of a mixture of several pure lipids, we first completely mixed the lipids by dissolution in $CHCl_3$ and then removed solvent under vacuum. The clear solution so prepared was exhaustively dialyzed against 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 6.6) at 4° (2–3 days). Vesicles were subsequently collected by centrifugation. Pellet composition was readily determined by analysis of the supernatant (phospholipid concentration was determined by phosphate assay (17); solid glycophorin was weighed into the initial solution and this solution was read at 280 nm in the presence of detergent before and after dialysis).

The 4-maleimido-derivative of Tempo [2,2,6,6-tetramethylpiperidine-1-oxyl, made according to the general procedure described by Griffith and McConnell (18)] was used to spin

Abbreviations: DSPC, distearoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; DPPE, dipalmitoylphosphatidylethanolamine.

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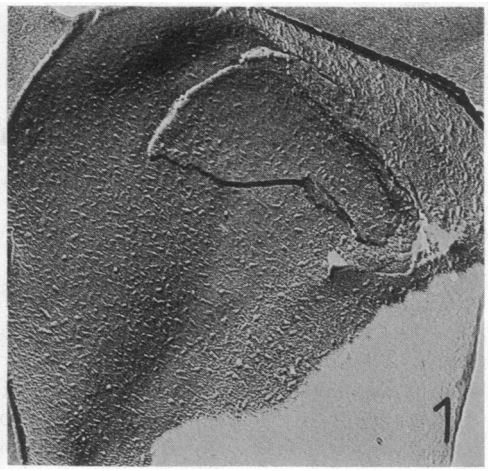

FIG. 1. Freeze-fracture micrograph of a lipid bilayer containing glycophorin in a molar ratio of about 120:1 lipid:glycoprotein. The lipid is 50/50 (weight ratio) phosphatidylserine/DMPC quenched from 23°. Note that the particles are often aggregates of smaller "bumps." (Unetched, ×80,000.)

label glycophorin. The labeling conditions were quite drastic (48 hr at 32° in pH 7.0 phosphate buffer) and no doubt involved nonspecific attack on several lysine residues. Unreacted label was removed by extensive dialysis at 4°. Gel electrophoresis of glycophorin labeled in this way with *N*-ethyl-[*1*-^{14}C]maleimide (New England Nuclear Corp.) proved the label to be associated only with the glycoprotein. Spin-labeled glycophorin could be incorporated into lipid by the same method used for unlabeled samples and the resulting systems were indistinguishable from unlabeled ones by freeze-fracture electron microscopy.

Influenza virus, strain A/PR8/34 (HONI), purified by erythrocyte adsorption and elution followed by preparative ultracentrifugation, was a gift of Dr. Alan P. Kendal of the University of Maryland Baltimore County (Department of Biological Sciences).

RESULTS

Glycophorin in Lipid Bilayers. Dialysis of dodecyltrimethylammonium bromide solutions of pure phosphatidylcholines such as DMPC, or of natural lipid extracts such as egg lecithin or erythrocyte lipid, leads to a population of bilayer vesicles which can range in size from about 250 Å up to several μm. Freeze-fracture electron micrographs of vesicles formed from extracted membrane lipids generally show fracture faces (bilayer hydrophobic region) and etch faces (bilayer surfaces) to be smooth. Certain pure phosphatidylcholines, on the other hand, display patterns on fracture and etch faces related to the phospholipid packing arrangements (19–25), and these patterns can sometimes be used to distinguish fluid from rigid lipid regions (24–26).

Fig. 1 shows a freeze-fracture electron micrograph of a vesicle of 50/50 weight ratio phosphatidylserine/DMPC containing glycophorin (lipid/protein mole ratio about 120:1). The fracture face particles result from incorporation of glycophorin. The etch faces (not shown here but shown in Fig. 5 for another lipid system) are smooth. Careful scrutiny of glycophorin-related particles in a variety of lipids, including extracted erythrocyte lipid, 50/50 phosphatidylserine/DMPC (weight ratio), 20/80 (mole ratio) cholesterol/DMPC, and 50/50 (mole ratio) DEPC/DPPC, often shows the particles to be short linear aggregates of up to five round bumps which

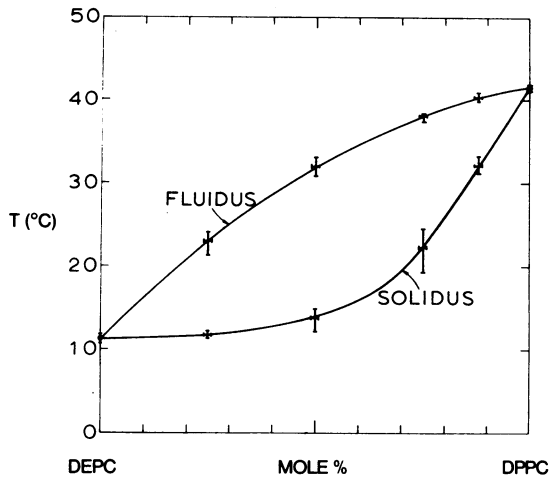

FIG. 2. Phase diagram for hydrated mixtures of DEPC and DPPC. This diagram was constructed from EPR measurements using the spin label Tempo. It enables one to predict the extent and composition of rigid–fluid lateral phase separations. Conditions corresponding to points above the fluidus represent totally fluid lipid mixtures while points below the solidus represent totally rigid lipid. For points between these two curves, fluid and rigid domains coexist.

are on the order of 40 Å in diameter (uncorrected for shadow thickness). Presumably the particles represent small clusters of glycoprotein and each "bump" is associated with one glycophorin molecule. In many cases the division between "bumps" in such aggregates is poorly resolved and the whole group then has the appearance of a single large, oblong bump. The existence of these fracture face particles would indicate that incorporated glycophorin penetrates the hydrophobic lipid region as it is thought to do in the intact erythrocyte.

Lipid Effects on Glycophorin Lateral Distribution. It is known that bilayers comprised of two different lipids can exhibit lateral phase separations in the plane of the membrane (27, 28). Lipid vesicles may be prepared from these systems which, in a certain temperature range, have regions of different composition and physical properties. As mentioned previously, certain pure phosphatidylcholines have characteristic surface patterns, and in these cases it is possible to distinguish fluid from rigid lipid by its appearance in freeze-fracture electron micrographs. (In this context "rigid" refers to "single component crystalline" or "solid solution crystalline" regions.) In binary mixtures of these lipids one can actually visualize the coexisting fluid and rigid domains and spin label-derived phase diagrams may be used to calculate the compositions of these regions (25).

We have examined the behavior of glycophorin in several systems of this type. For instance a phase diagram has been derived for the binary system of hydrated lipids, DEPC–DPPC and has been checked at various compositions and temperatures by freeze-fracture electron microscopy (25). This phase diagram is shown in Fig. 2 and may be used to predict the extent and composition of temperature-induced rigid–fluid lateral phase separations in mixtures of DEPC and DPPC. Lipid systems corresponding to points above the fluidus curve are totally fluid (jumbled freeze-fracture appearance), while those corresponding to points below the solidus are totally rigid (ordered freeze-fracture appearance). Points in between these curves represent lipid mixtures having coexisting fluid and rigid regions. At 23° a 50/50

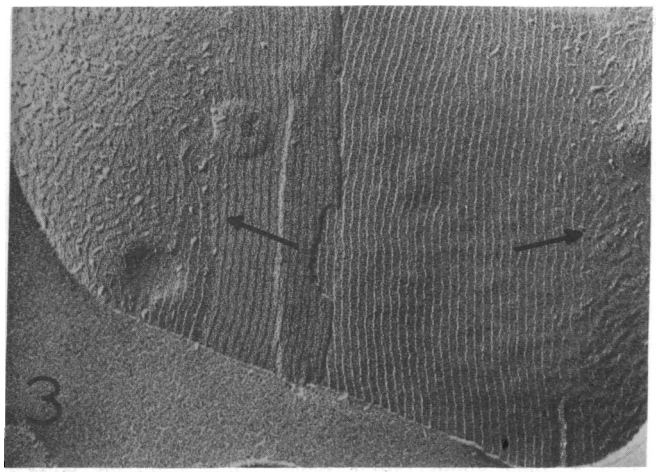

FIG. 3. Freeze-fracture micrograph of 50/50 mole % DEPC–DPPC with incorporated glycophorin. The lower half of a large vesicle is shown. Arrows indicate boundaries between fluid lipid domains containing glycophorin-related particles (far left and far right) and a rigid lipid domain (center with ordered lines). The sample was quenched from 23°, a temperature at which the phase diagram in Fig. 2 predicts roughly equal areas of fluid and rigid lipid. (Unetched, ×80,000).

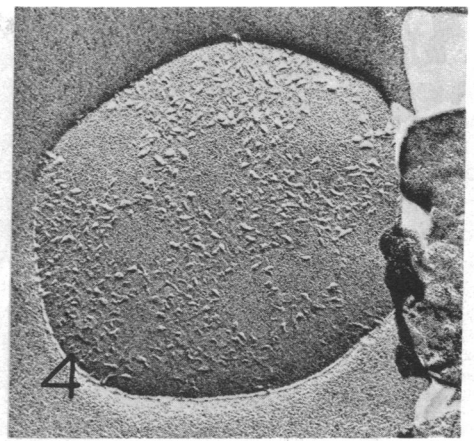

FIG. 4. Platinum–carbon replica of a vesicle of 35/65 mole % DPPE–DEPC containing glycophorin and quenched from a temperature (52°) 1 or 2° above the point at which rigid lipid domains should begin to appear. It is likely that the roughly circular patches void of particles result from inadequately rapid sampling quenching (glycoprotein is excluded from the lipid as it freezes). (Unetched, ×59,000.)

(mole ratio) mixture has roughly equal areas of jumbled (fluid) and ordered (rigid) surface patterning. Fig. 3 shows a 50/50 DEPC–DPPC lipid bilayer containing glycophorin which was equilibrated at 23° prior to being rapidly quenched in Freon and freeze-fractured. Note that the glycophorin-related particles are localized in fluid regions of the bilayer. This same general observation was made in 50/50 DMPC–DSPC lipid bilayers at 36° [a set of conditions under which the spin label-derived phase diagram predicts coexistence of fluid and rigid domains (27)]. It would appear likely that this may be a general rule: that the glycophorin–lipid complex, given a choice, will associate with fluid lipid regions.

The response time for the process of protein lateral distribution shift can be quite rapid. Fig. 4 shows a freeze-fracture electron micrograph of 35/65 (mole ratio) DPPE–DEPC quenched from a degree or two *above* the onset temperature for a rigid–fluid lipid phase separation (S. H. Wu and H. M. McConnell, unpublished experiments). This lipid system has no dramatic surface patterns indicative of the state of the lipid. However, the circular clear patches appear to be a result of the finite time (a fraction of a second) required for the sample to pass from its incubation temperature to a completely immobilized state when plunged into liquid Freon cooled in liquid nitrogen. This situation is very similar to that recently reported (26, 29) in a β-oxidation deficient fatty acid auxotroph of *Escherichia coli* with a simple inner membrane lipid composition (these showed circular patches void of particles when quenched from their growth temperature of 37° unless previously fixed in glutaraldehyde). It would appear that the rigidifying lipid excludes protein as it freezes outward from centers of crystallization (the centers of the clear patches).

Incorporated Glycophorin As a Receptor Site. Glycophorin, by virtue of its exposed sialic acid residues, is the human erythrocyte influenza virus receptor. Erythrocyte cross-linking via these receptors forms the basis for the influenza virus hemagglutination assay. This assay also works with the model systems described here.

Lipid vesicles of 50/50 (weight ratio) DEPC–DPPC or egg lecithin were generated by dialysis with and without glycoprotein in molar ratios of 700:1 to 120:1 (lipid:glycoprotein) and washed repeatedly by low-speed centrifugation (1000 × *g*) in Veronal-buffered saline (pH 7.0). This washing procedure removed traces of unincorporated glycophorin and ensured that the vesicles were large enough to settle at a rate comparable to that of intact erythrocytes. Vesicles prepared in this way containing glycophorin: (*a*) competed successfully with erythrocytes for influenza virus in viral hemagglutination inhibition test, (*b*) were cross-linked by influenza virus and settled in a manner analogous to that of erythrocytes.

Fig. 5 is a micrograph of a freeze-etched preparation of 50/50 DEPC–DPPC vesicles containing glycophorin (about 700:1 lipid:glycoprotein molar ratio) which have been aggregated by influenza virus. The viral neuraminidase had been previously inactivated by heating at 56° for 30 min. Following agglutination in Veronal-buffered saline the preparation was washed in 10 mM phosphate buffer (pH 7.0) at room temperature (23°) and quenched in Freon 22 cooled in liquid nitrogen. The micrograph shows two vesicles whose fluid regions (containing glycophorin particles and pits caused by etching) are joined by virus particles. For comparison, similar electron microscopy studies were done on erythrocytes with influenza virus. Note that the regular pattern on the fracture face of the lower vesicle matches precisely with that on the etch face (fluid regions also match on both faces). In general, adjacent monolayers comprising the bilayer appear to be in the same state and are therefore in effective communication.

Effect of Glycophorin on the Lipid. The characteristic temperature at which the phospholipid acyl chains in pure DMPC bilayers undergo a transition from the highly conformationally mobile state to a rigid, all-*trans* configuration is 23.5°‡. This value has been measured using the spin label Tempo on

‡ Values of 23.2° and 23.7° also have been reported in the literature (27, 30).

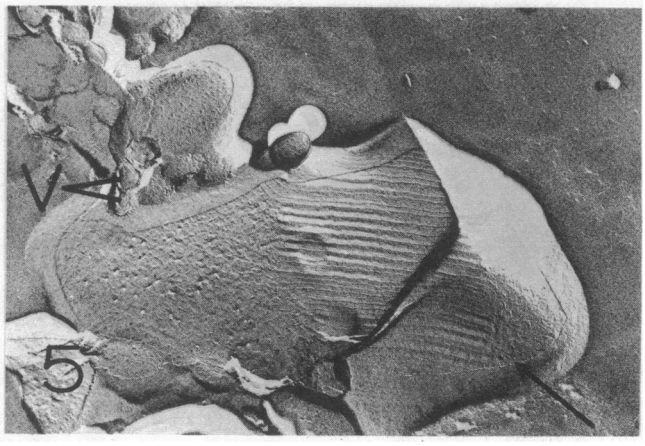

FIG. 5. Freeze-etched preparation of 50/50 (weight ratio) DEPC–DPPC vesicles containing glycophorin. They have been agglutinated by influenza virus at 23° (fluid and rigid lipid regions coexist). A large, oblong vesicle is shown which possesses fluid domains at each end and a rigid, orderly patterned region in the center. The fluid regions contain particles (and some pits caused by etching). The arrow at lower right indicates the boundary between fracture and etch faces. Note that adjacent fracture and etch face regions seem to be in the same state of fluidity (i.e., both halves of the bilayer). A smaller vesicle is shown bound to the upper left hand etch face of the large vesicle by at least five viruses (two of these have been fractured and are indicated by V and three more, just to the right, have been exposed by etching). Note that the aggregation points appear to be fluid regions containing glycophorin. (Etched 45 sec at −106°, ×40,000.)

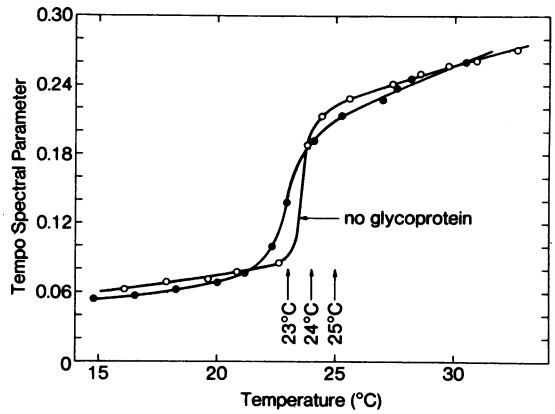

FIG. 6. Plots of the Tempo spectral parameter [which is related to lipid fluidity (27)] versus temperature for DMPC vesicles with (●) and without (○) glycophorin. The sample with glycophorin was 120:1 mole ratio lipid:glycoprotein. The inflection points in the plots indicate the fluid–rigid lipid transition. These particular experiments were run with descending temperature.

vesicles generated by dialysis from dodecyltrimethylammonium bromide. The Tempo spectral parameter (27) is shown plotted in Fig. 6. The addition of glycophorin in a molar ratio of 120:1 (lipid:glycoprotein) had the effect of broadening the rigid–fluid transition and of shifting it to a slightly lower temperature (Fig. 6). This is the effect one expects to see upon introduction of an impurity into a pure substance when the impurity dissolves exclusively in the fluid phase. If glycophorin and fluid DMPC formed an ideal solution, the calculated lowering of the melting temperature is about 0.3° [this has been calculated using the transition enthalpy ΔH = 6.26 kcal (26.2 kJ)/mol given by Hinz and Sturtevant (30)].

The Tempo spin label technique reflects bulk lipid behavior and does not necessarily faithfully record the behavior of lipid directly in contact with glycophorin. The electron paramagnetic resonance spectrum of glycophorin covalently labeled with the maleimide derivative of Tempo is to some extent sensitive to the lipid environment of the reincorporated glycoprotein. Spin-labeled glycophorin responds to the DMPC phase transition; however, the response is relatively small, presumably because (*a*) the labeling sites are not intimately associated with lipid and/or (*b*) the spin label spectral features are due mainly to its immobilization by covalent attachment to glycophorin and the lipid effects are small in comparison. Interestingly, the DMPC transition temperature measured by plotting changes in the spectrum of maleimide spin-labeled glycophorin (e.g., peak height of the more mobile component) is about 24.8° (or 1.5–2.0° *higher* than the bulk lipid transition in DMPC bilayers containing the same amount of unlabeled glycophorin). Presumably this reflects the fact that, in the case of DMPC at least, as the temperature is lowered, the phospholipid around glycophorin becomes immobilized somewhat more readily than that in the bulk lipid. That the observed inflection in spectral parameter plots is indeed related to the lipid, DMPC, is shown by the fact that there is no trace of an inflection point in spectral feature plots for labeled glycophorin in 50/50 (mole ratio) DEPC–DPPC between 33° and 13°. However, there is a slight change of slope at the former temperature and a larger change at the latter (these temperatures correspond to the onset and completion of a lateral phase separation, as shown in Fig. 2).

DISCUSSION

In the past few years considerable progress has been made in the isolation of membrane macromolecules. This raises the possibility of studying membrane biochemistry by examining the functions of individual components in the manner that has proven so successful with cytoplasmic systems. However, many membrane components are intimately tied both structurally and functionally to membrane lipid. It is this fact that has led various groups in recent years to investigate the incorporation of purified membrane proteins into lipid bilayers. Glycophorin is the first glycoprotein to be studied in this manner. Tosteson *et al.* (31) have added glycophorin to the aqueous compartments in black lipid film experiments and reported that it associates with the films and affects their conductance. It has been our experience that simply adding glycophorin to an aqueous suspension of vesicles fails to produce the particles seen in freeze-fracture electron micrographs of glycophorin-containing vesicles generated by dialysis.

The particles seen by freeze-fracture electron microscopy in biological membrane fracture faces are generally agreed to represent points at which membrane proteins penetrate the hydrophobic lipid region. In the case of the human erythrocyte there are two (glyco-) proteins known to do this: glycophorin and protein III [also known as "100,000 molecular weight protein" and "component a" (4, 32)]. It has been postulated that these two glycoproteins may associate with one another and together form the now classic 85-Å membrane particle of human erythrocytes (33). Both of these hypotheses are consistent with our observation that glyco-

phorin alone forms smaller particles in lipid bilayers. In addition to its proposed association with protein III, it is thought that glycophorin interacts, either directly or indirectly, with the spectrin network on the inner surface of erythrocytes (10). The lack of one or both of these components may lead to the clustering of glycophorin with itself in lipid bilayers.

Stier and Stackman, in studying the cytochrome *P*450 reductase hydroxylating enzyme system of rabbit liver microsomal membranes, found evidence that a rather rigid "phospholipid halo," distinct from the bulk lipid, exists around the protein (34). It is known that several isolated membrane proteins [cytochrome oxidase (35) and cytochrome b_5 (36)] can interact strongly with (and immobilize) bound lipid. Our experiments with glycophorin in DMPC indicate that lipid in direct contact with the glycoprotein is more readily immobilized than the bulk lipid. Paradoxically, however, the entire lipid–protein complex (the fracture face particle) tends to associate with fluid regions when a choice exists. W. Kleemann of this laboratory has found that the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum shows a similar behavior (26) in that this protein tends to be localized in fluid lipid regions.

Simple correlations between lipid phase diagrams and freeze-fracture microphotographs such as those illustrated in Figs. 2 and 3 (and 5) are due to a fortunate combination of circumstances that are not always achieved experimentally. For example, our ability to visualize rigid domains of phosphatidylcholines is due to their "banded" appearance. These bands are not exhibited by all rigid lipids, or rigid lipid mixtures. The ability to trap the ambient temperature domain structure also appears to be limited to certain lipid systems. It has been noted here, and elsewhere (29), that membranes containing phosphatidylethanolamine apparently crystallize so rapidly during temperature quenching that the observed fracture patterns correspond to temperatures below the ambient temperature. Finally, the preference of membrane proteins for the fluid lipid phase when fluid and rigid lipid domains coexist in equilibrium may also be limited to certain lipids and proteins. For example, if coexisting fluid and rigid lipid domains have sufficiently similar physical properties, intrinsic proteins might be found in both domains.

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