Photoactivatable Carbene-Generating Phospholipids: Physical Properties and Use in Detection of Phase Separations in Lipid Mixtures†

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ABSTRACT: Phospholipids which carry photoreactive carbene-generating groups have been cross-linked with a variety of acceptor phospholipids in binary and ternary mixtures, and the results have been correlated with the physical behavior of the lipids involved. The photoreactive phospholipids 1-palmitoyl-2-[ω-(2′-diazo-3′,3′,3′-trifluoropropionoxy)lauroyl]-phosphatidylcholine (PCI) and 1-palmitoyl-2-[ω-(*m*-diazirinophenoxy)undecanoyl]phosphatidylcholine (PCII) undergo characteristic acyl chain order–disorder transitions at ~17 and ~6 °C, respectively, as demonstrated by differential scanning calorimetry. In equimolar mixtures of these lipids with dipalmitoyllecithin (DPL), phase separation occurs below characteristic temperatures as has been previously observed for mixtures of saturated and unsaturated phospholipids. Photolysis of mixtures of PCI or PCII with saturated or unsaturated phospholipids results in the formation of covalently linked phospholipid dimers. Photolysis-dependent dimer formation was studied in mixtures of DPL and dioleyllecithin (DOL) which exhibit temperature-dependent phase separation and also in mixtures of DPL and dimyristoyllecithin (DML) which do not exhibit phase separation. Covalent cross-linking of PCII to each of the lipids in these mixtures was quantitated as a function of temperature. The photoreactive phospholipid was excluded from gel phases in both the DPL/DOL and DPL/DML systems and reacted preferentially with the liquid-crystalline phospholipid in the phase-separated DPL/DOL system. Thus, the photochemical labeling approach is sensitive to the presence of phase separations and has the capability of identifying the major components of each phase.

Despite extensive studies of membrane structure by a variety of approaches, certain aspects of the organization of proteins and lipids in membranes remain poorly understood. For example, virtually nothing is known at present about the organization in the membrane plane, i.e., (1) whether particular lipid classes surround particular membrane proteins and (2) whether membrane lipids not associated with proteins are homogeneously mixed in the membrane plane or clustered into functional units. Recently, in an approach to the problems of lipid–lipid and lipid–protein interactions in membranes, a series of phosphatidylcholines which carry photoactivable groups at the ω position of the *sn*-2 acyl chain has been synthesized (Gupta et al., 1977). The carbene-generating precursors used are the trifluorodiazopropionyloxy (Chowdhry et al., 1976) and diazirinophenoxy groups (Smith & Knowles, 1975) (Figure 1), both of which can be photolyzed at wavelengths of ~360 nm, thus avoiding ultraviolet photodamage to aromatic amino acid residues of proteins. The photogenerated carbenes have been shown to insert into C–H bonds of fatty acyl chains (Gupta et al., 1979; Radhakrishnan et al., 1980).

Sonicated vesicles composed of pure photoreactive phospholipids trap small solutes and have an internal volume similar to those observed for natural phospholipids (C. M. Gupta, R. Radhakrishnan, and H. G. Khorana, unpublished experiments). In addition, lipid–lipid cross-linking studies indicate that the intermolecular cross-linking occurs in the acyl chain region, with no cross-linking to the glycerol backbone or choline head groups (Gupta et al., 1979). Therefore, the carbene-generating group at the fatty acyl ω position does not make excursions to the bilayer surface. In the present work experiments have been carried out to determine whether these photoreactive phosphatidylcholines exhibit physical properties similar to those of natural phospholipids and how they interact with other phospholipids. Differential scanning calorimetry (DSC)[1] shows that the photolabeled phospholipids PCI and PCII (Figure 1) exhibit behavior typical of synthetic phospholipids, with order–disorder transition temperatures (T_M's) which are lower than those of the corresponding saturated phosphatidylcholines. Photolysis-dependent cross-linking to form phospholipid dimers has been studied as a function of temperature for a variety of phospholipid mixtures which either exhibit or do not exhibit lateral phase separations. Under conditions in which fluid and solid bulk lipid phases coexist, the photolabeled phospholipid preferentially reacts with the major component of the fluid phase, demonstrating that the photolabeled phospholipid partitions into the fluid phase and is partially excluded from the solid phase. Under conditions in which a single solid bulk phase exists, the photolabeled phospholipid is partially excluded. In mixtures of saturated and unsaturated phosphatidylcholines above the bulk phase separation temperature, the photogenerated carbene reacts preferentially with the unsaturated phospholipid, presumably due to preferential reaction of the electrophilic carbene with double bonds. However, photogenerated carbenes are not scavenged by the olefinic chains, since reaction with the saturated acyl chains is also observed. These results indicate that the photochemical cross-linking approach can be used to monitor phospholipid phase separations and to identify the

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[1] Abbreviations used: DSC, differential scanning calorimetry; DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; DOL, dioleyllecithin; [^{14}C]DPL, di[1-^{14}C]palmitoyllecithin; [^{14}C]DOL, di[1-^{14}C]oleyllecithin; [^{14}C]DML, [*N-methyl*-^{14}C]DML; T_M, acyl chain order–disorder transition temperature; PCI, 1-palmitoyl-2-[ω-(2′-diazo-3′,3′,3′-trifluoropropionoxy)lauroyl]phosphatidylcholine; PCII, 1-palmitoyl-2-[ω-(*m*-diazirinophenoxy)undecanoyl]phosphatidylcholine; TLC, thin-layer chromatography.

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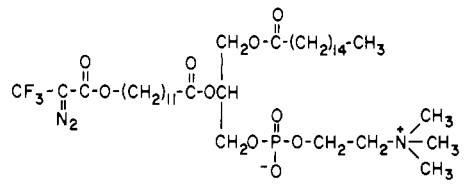
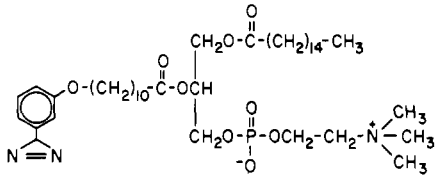


PC I



PC II

FIGURE 1: Synthetic photolabeled phospholipids. (I) 1-Palmitoyl-2-[ω-(2′-diazo-3′,3′,3′-trifluoropropionoxy)lauroyl]phosphatidylcholine (PCI); (II) 1-palmitoyl-2-[ω-(*m*-diazirinophenoxy)undecanoyl]-phosphatidylcholine (PCII).

major components of each phase.

Materials and Methods

Materials. Oleic acid and palmitic acid were purchased from Nu-Check Prep, Inc. (Elysian, MN). [1-^{14}C]Oleic acid, [1-^{14}C]palmitic acid, and [^{14}C]methyl iodide were from New England Nuclear (Boston, MA). Dimyristoylphosphatidylethanolamine and dimyristoyllecithin (DML) were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were reagent grade.

Synthetic Phospholipids. Dioleyllecithin (DOL), dipalmitoyllecithin (DPL), di[1-^{14}C]oleyllecithin ([^{14}C]DOL), and di[1-^{14}C]palmitoyllecithin ([^{14}C]DPL) were synthesized as previously described (Gupta et al., 1977). [*N-methyl*-^{14}C]Dimyristoyllecithin ([^{14}C]DML) was synthesized from dimyristoylphosphatidylethanolamine and [^{14}C]methyl iodide by Dr. A. Ross of this laboratory by a modification of published procedures (Stoffel, 1975). 1-Palmitoyl-2-[ω-(2′-diazo-3′,3′,3′-trifluoropropionoxy)lauroyl]phosphatidylcholine (PCI) and 1-palmitoyl-2-[ω-(*m*-diazirinophenoxy)undecanoyl]phosphatidylcholine (PCII) were synthesized as previously described (Gupta et al., 1977). [*N-methyl*-^{14}C]PCI was synthesized by chemical demethylation of PCI followed by remethylation with [^{14}C]methyl iodide as described above.

Lipid Dispersions. Appropriate quantities of various lipids were dissolved in $CHCl_3$ or in $CHCl_3$–CH_3OH (2:1), the solutions were dried under N_2, and residual films were desiccated in a vacuum. Water was added and the mixture was vortexed at a temperature above the lipid order–disorder transition temperature. In some cases the dispersions were sonicated under N_2 for ~30 min in a bath-type sonicator at a temperature above T_M. All experiments were performed on unsonicated multilamellar dispersions unless otherwise indicated.

Differential Scanning Calorimetry. Mixtures of lipids were dried from their solutions in the above organic solvents in stainless steel pans (50-μL capacity) and desiccated in a vacuum. The pans were sealed after addition of water. The final lipid concentration was ~10% by weight. The thermal behavior of the dispersions was determined by using a Perkin-Elmer DSC-2 differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, CT) at a heating/cooling rate of 5 °C/min.

Photolysis and Analysis. Phospholipid dispersions were irradiated at 366 nm with an Oriel Hg–Xe arc lamp (Oriel Corp., Stamford, CT) with a monochromator and two filters

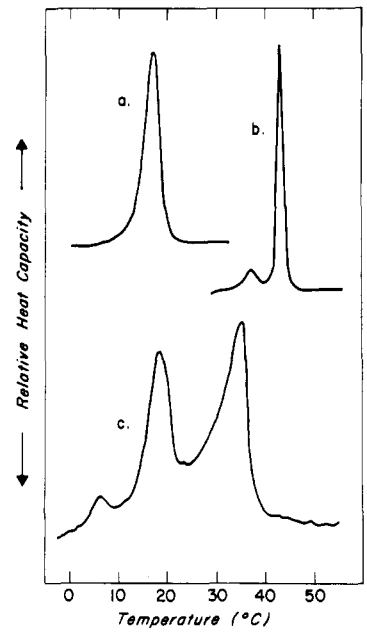


FIGURE 2: Differential scanning calorimetry traces of unsonicated aqueous dispersions of (a) PCI, (b) DPL, and (c) 1:1 PCI/DPL (mol/mol).

(WG360, WG345, Schott Optical, Duryea, PA). Samples were irradiated under N_2 for a period of time sufficient to ensure complete photolysis of either the diazo or the diazirine groups (1 h or 20 min for PCI and PCII, respectively).

Irradiated samples were extracted (Bligh & Dyer, 1959), and the organic solvent extract was chromatographed on plastic-backed TLC plates in $CHCl_3$–CH_3OH–H_2O (65:25:4). After visualization by radioautography, phospholipid monomer and dimer spots were cut out and counted in a liquid scintillation counter. No dimeric material was observed in samples which were not irradiated or in samples containing saturated and/or unsaturated phospholipids irradiated in the absence of photolabeled phospholipid.

Results

Studies with 1-Palmitoyl-2-[ω-(2′-diazo-3′,3′,3′-trifluoropropionoxy)lauroyl]phosphatidylcholine (PCI). A DSC trace of an aqueous dispersion of PCI is shown in Figure 2. The effects of incorporating the ω-(2′-diazo-3′,3′,3′-trifluoropropionoxy)lauroyl chain in the *sn*-2 position of the phospholipid are a decrease in the T_M by ~25 °C (compared to that of DPL) and a small broadening of the transition temperature range. The transition enthalpy is 10.7 kcal/mol. A DSC trace of an equimolar mixture of PCI and DPL is also shown in Figure 2. In this case two peaks are observed: a relatively sharp transition at ~18 °C and a broad transition at ~34 °C. This behavior indicates that on cooling a phase separation occurs at ~34 °C, resulting in a PCI-rich liquid crystalline phase and a DPL-rich gel phase. This phase separation occurs at a temperature which is reduced compared with that of the order–disorder transition of pure DPL because of the presence of PCI in DPL. As the temperature is lowered through the transition at ~18 °C, the PCI-rich phase solidifies into the gel state. A minor transition is also observed at ~7 °C.

Photochemical cross-linking of mixtures of PCI and DPL was studied in the light of the above-described DSC behavior. Vortexed aqueous dispersions of equimolar amounts of PCI and DPL were photolyzed, and the products were analyzed by thin-layer chromatography. Covalent cross-linking of PCI to DPL and total cross-linking were determined in separate

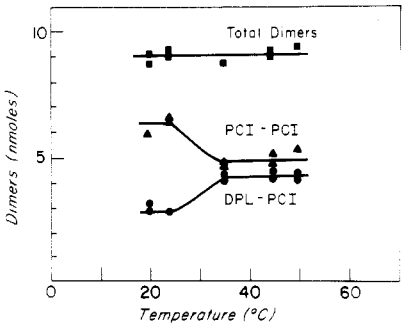


FIGURE 3: Temperature dependence of cross-linking of an equimolar mixture of PCI and DPL (unsonicated).

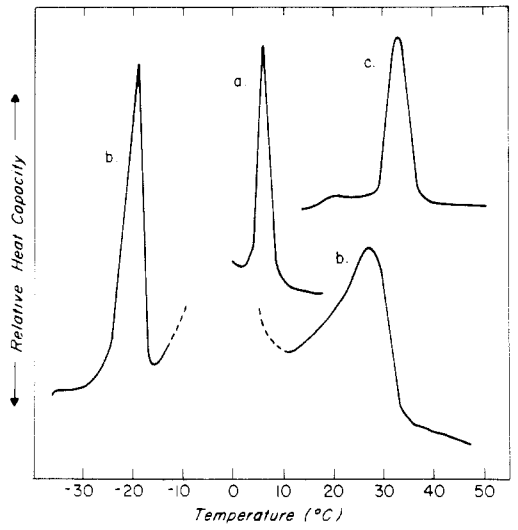


FIGURE 4: Differential scanning calorimetry traces of unsonicated dispersions of (a) PCII, (b) 1:1 DOL/DPL (mol/mol), and (c) 1:1 DML/DPL (mol/mol).

experiments in which a ^{14}C label was present in DPL or PCI, respectively. Figure 3 shows that the total cross-linking observed is constant over the temperature range 19–49 °C. Cross-linking of PCI to DPL, however, shows a decrease at temperatures below ~30 °C, while the extent of PCI–PCI dimer formation increases at temperatures below ~30 °C. These results are consistent with the interpretation that the local concentration of PCI decreases in the vicinity of DPL as the temperature is lowered below ~30 °C, as predicted by the DSC results.

Studies with 1-Palmitoyl-2-[ω-(m-diazirinophenoxy)undecanoyl]phosphatidylcholine (PCII). Differential scanning calorimetry of an aqueous dispersion of PCII reveals a sharp acyl chain order–disorder transition at ~6 °C (Figure 4). Thus, the major effect of replacing the palmitate in the *sn*-2 position of DPL with an ω-(*m*-diazirinophenoxy)undecanoyl chain is a decrease in the phospholipid T_M by ~36 °C. The transition enthalpy is 5.9 kcal/mol. An equimolar mixture of PCII and DPL exhibits a temperature-dependent phase separation similar to that described above for PCI/DPL mixtures (data not shown).

The potential use of PCII to detect bulk lipid phase separation was investigated by studying the temperature dependence of cross-linking in two model lipid systems, one of which exhibits a phase separation while the second does not. The calorimetric behavior of an equimolar mixture of DPL and DOL is shown in Figure 4. Two transitions are observed: a relatively sharp peak at −20 °C and a broad peak at ~30 °C. The dashed peak at 0 °C is the high-enthalpy ice–water transition, which is off scale at this sensitivity. The double

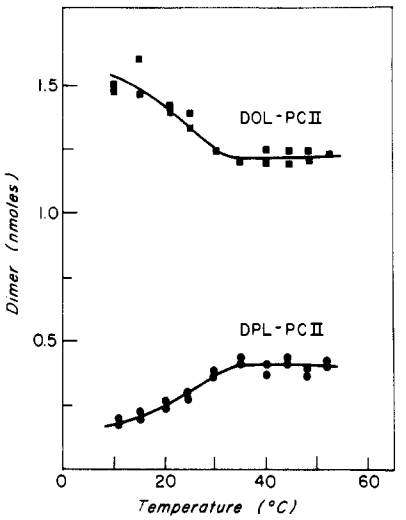


FIGURE 5: Temperature dependence of cross-linking of an equimolar mixture of DOL and DPL containing 10 mol % PCII (unsonicated).

peak indicates that a phase separation occurs in this system. Below the 30 °C transition, a DOL-rich liquid-crystalline phase and a DPL-rich gel phase coexist. Below the −20 °C transition, the DOL-rich phase enters the gel state. This observation is in agreement with a previously published report (Ladbrooke & Chapman, 1969).

Vortexed aqueous dispersions composed of 90 mol % 1:1 [^{14}C]DPL/DOL and 10 mol % PCII were photolyzed at a variety of temperatures. Identical mixtures with [^{14}C]DOL and unlabeled DPL were also photolyzed. The temperature dependence of formation of covalently cross-linked DPL–PCII and DOL–PCII dimers is shown in Figure 5. The absolute magnitude of cross-linking of PCII to DOL is higher than that to DPL at all temperatures studied. At temperatures greater than ~30 °C, cross-linking to both DPL and DOL is constant with temperature. As the temperature is lowered below ~30 °C, cross-linking to DOL increases while cross-linking to DPL decreases. The results indicate that, at temperatures below ~30 °C, PCII partitions into the more fluid DOL-rich phase and is partially excluded from the solid DPL-rich phase. This result is consistent with the observation that the T_M of PCII (6 °C) is lower than the DPL/DOL phase separation temperature (~30 °C). Thus, the photochemical cross-linking method is capable of detecting bulk phase separations. In addition, this approach detects which lipid has gone into which phase, i.e., that the fluid phase is DOL-rich and the solid phase is DPL-rich.

Similar experiments were performed with an equimolar mixture of DPL and DML, a mixture which exhibits complete miscibility in both the gel and liquid-crystalline states (Shimshick & McConnell, 1973; Chapman et al., 1974). In Figure 4 is shown a DSC trace of an equimolar mixture of DML and DPL. A single order–disorder transition is observed at 32 °C, a temperature midway between that of pure DML (22 °C) and pure DPL (41 °C). Vortexed aqueous dispersions composed of 90 mol % 1:1 [^{14}C]DPL/DML and 10 mol % PCII were photolyzed at a variety of temperatures. The experiment was repeated with [^{14}C]DML and unlabeled DPL. The temperature dependence of covalent cross-linking of PCII to DML and to DPL is shown in Figure 6. The extent of cross-linking to DML and DPL was invariant over the temperature range ~30–50 °C. At temperatures below ~30 °C, cross-linking to both DML and DPL decreased, in contrast to the situation observed with DOL/DPL mixtures. This result is consistent with partial exclusion of PCII from the DML/

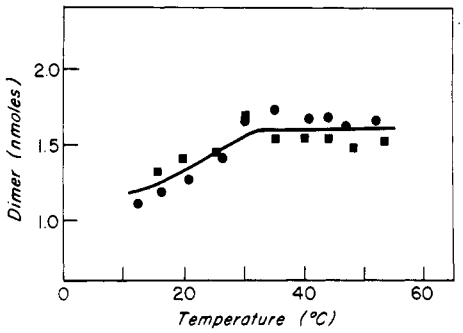


FIGURE 6: Temperature dependence of cross-linking of an equimolar mixture of DML and DPL, containing 10 mol % PCII: (●) PCII–DPL dimers; (■) PCII–DML dimers (unsonicated).

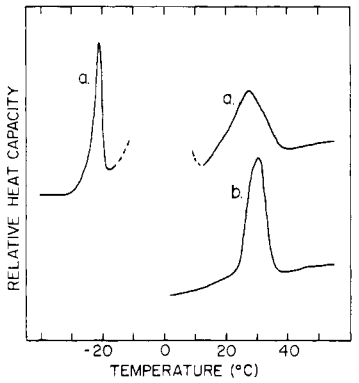


FIGURE 7: Differential scanning calorimetry traces of unsonicated aqueous dispersions composed of (a) 90 mol % 1:1 DPL/DOL and 10 mol % PCII and (b) 90 mol % 1:1 DPL/DML and 10 mol % PCII.

DPL mixture, which is in the gel state below ~30 °C. Again, this is consistent with the observation that the T_M for PCII (6 °C) is lower than that of the bulk DML/DPL mixture (~32 °C).

A major consideration in such studies is the extent to which the ω-diazirinophenoxy group perturbs the phase behavior of the system under study. Thus we have obtained DSC traces of lipid mixtures identical with those with which the cross-linking experiments were performed. Figure 7a shows a DSC trace of an aqueous dispersion composed of 90 mol % 1:1 DPL/DOL and 10 mol % PCII. Minor changes are observed compared with 1:1 DPL/DOL: a 1–2 °C decrease in the peak temperature of the low-temperature transition and perhaps a slight broadening of the higher temperature phase separation transition at ~30 °C. In Figure 7b is shown a DSC trace for a dispersion composed of 90 mol % 1:1 DML/DPL and 10 mol % PCII. Compared with 1:1 DML/DPL, the peak maximum is decreased by ~2 °C, and the transition width is broadened.

Preference of Photogenerated Carbene for Unsaturated Acyl Chains. The results of cross-linking experiments with DOL, DPL, and PCII indicated a 2-fold higher formation of DOL–PCII dimers over DPL–PCII dimers (Figure 5). No significant preference was observed between cross-linking to DML or DPL (Figure 6). For further exploration of this preference for DOL, cross-linking experiments were performed on sonicated vesicles composed of various combinations of PCII, DOL, and DPL. The molar ratio of PCII to nonphotolabeled lipid was 1:1 in all cases. The relative amounts of DOL and DPL were varied, and cross-linking to each was determined by using either [^{14}C]DOL or [^{14}C]DPL. Figure 8 shows that there is an ~2-fold preference for reaction with DOL. However, DOL does not scavenge photogenerated carbenes; i.e., cross-linking to DPL is still observed in the presence of DOL. An identical experiment was performed with the dia-

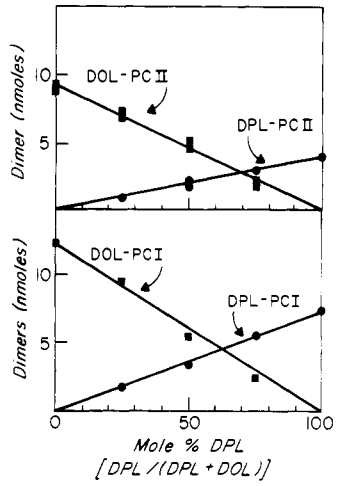


FIGURE 8: Cross-linking of PCII (top panel) or PCI (bottom panel) to DPL and DOL at 47 °C in sonicated dispersions of varying DPL/DOL ratio. The mole ratio of photoactive phospholipid to (DPL plus DOL) is 1.0.

zophospholipid, PCI. Figure 8 shows that although there is an ~2-fold preference of PCI for the unsaturated lipid, the photogenerated carbenes are again not scavenged by DOL.

Discussion

Differential scanning calorimetry of pure phospholipids carrying the trifluorodiazo and phenoxydiazirine groups indicates that these compounds exhibit temperature-dependent behavior typical of phosphatidylcholines. The presence of these photoactivable groups in the ω position of the phosphatidylcholine *sn*-2 chain results in a decrease in the temperature of the order–disorder transition compared with that of the parent saturated lecithins. In equimolar mixtures with DPL, the trifluorodiazo lipid PCI undergoes phase separation below a characteristic temperature, as has been observed for mixtures of saturated and unsaturated lecithins (Ladbrooke & Chapman, 1969). A similar phase separation is also observed for an equimolar mixture of the diazirino lipid PCII with DPL.

Photolysis of mixtures of PCI or PCII with saturated or unsaturated phospholipids results in covalent cross-linking. The quantitative distribution of the various types of dimer formed is consistent with the physical properties of the phospholipid mixtures as determined by calorimetry. For example, in 1:1 mixtures of PCI with DPL, the extent of DPL–PCI dimer formation decreases at temperatures below the calorimetrically defined phase separation temperature. The total quantity of dimers produced (DPL–PCI and PCI–PCI) is insensitive to the phase separation in this PCI/DPL system. In ternary mixtures in which the photolabeled lipid is a minor component, cross-linking data indicate that the photolabeled lipid is partially excluded from the gel phase of the bulk lipid. Thus, cross-linking of PCII to DPL in a DPL/DOL/PCII mixture (1:1:0.2 mol/mol/mol) decreases at temperatures below the calorimetrically determined bulk phase separation temperature (Figure 5). Under the same conditions, cross-linking of PCII to DOL increases, indicating that the local concentration of PCII is higher in the DOL-rich liquid-crystalline phase than in the DPL-rich gel phase. Thus, the photochemical cross-linking approach can identify which lipid is in which phase and can be used to provide microscopic information about the composition of separated phases in complex phospholipid mixtures and in membranes.

The presence of the ω-diazirinophenoxy group in the center of the bilayer is expected to perturb the local lipid environment to some extent. Such perturbation has been previously de-

scribed for both ESR spin-labels and fluorescent probes (Cadenhead & Müller-Landau, 1976; Cadenhead et al., 1977). By being covalently linked to the ω position of a phospholipid acyl chain, the photolabel is constrained to remain primarily in the central region of the bilayer, where perturbations are more easily accommodated (Cadenhead et al., 1977). Figure 7b demonstrates that the presence of 10 mol % PCII results in a broadening of the transition width of the DML/DPL system, in addition to a 2 °C decrease in the transition maximum. The effects of 10 mol % PCII on the DPL/DOL system (Figure 7a) are also minimal, with no significant effect on the broad phase separation at $\sim$30 °C. Thus, the general features of the bulk phase behavior in these simple binary systems are not significantly altered by the presence of PCII at the 10% level. We expect that small quantities of PCII would cause insignificant perturbation of the bulk phase behavior of natural lipid mixtures, which exhibit broad phase transitions.

When a saturated and an unsaturated phospholipid compete as cross-linking acceptors with either the trifluorodiazo or diazirino lipids, a preference is observed for cross-linking to the unsaturated lipid. This preference is presumably due to preferential reaction of electrophilic carbene with double bonds but could also be due to a specific interaction of the photolabel with the kink at the double bond in the unsaturated acyl chain. The latter explanation is unlikely since the preference for reaction with unsaturated lipid can be observed above the phase separation temperature in sonicated vesicles in which the unsaturated phospholipid is diluted with saturated phospholipid. It is important to note that the unsaturated lipid does not scavenge carbenes; i.e., cross-linking to saturated lipid is still observed in the presence of unsaturated lipid.

The approach which we have taken provides qualitative information on the distribution of lipids between gel and liquid-crystalline phases. In theory, the photo-cross-linking method can provide quantitative information on the amount of each lipid present in each phase. For this purpose, knowledge of the efficiency of cross-linking to each type of lipid acyl chain and determination of complete phase diagrams using the photo-cross-linking method would be required. In practice, this is probably difficult to attain. On the other hand, the strength of the photolabeling approach will lie in the ability to obtain qualitative information about complex systems rather than as a quantitative chemical means of analyzing simpler phase diagrams.

In conclusion, the physical behavior of the carbene-generating photolabeled phospholipids is consistent with current knowledge of phospholipid behavior in model systems. These compounds can detect phase separations in simple model lipid systems and, in addition, can be used to determine the major components of each phase. It is hoped that these photolabeled phospholipids will ultimately provide previously unavailable microscopic information about the lateral and transverse organization of biological membranes.

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