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Cross-linking of phosphatidylethanolamine neighbors with dimethylsuberimidate is sensitive to the lipid phase

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Dimethylsuberimidate was reacted with aqueous dispersions of dipalmitoylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, dilauroylphosphatidylethanolamine, and dielaidoylphosphatidylethanolamine at pH 10 and at pH 8. The amount of amidine dimer formation was about four times greater above the gel-to-fluid phase transition of each lipid than below the transition. The transition temperature of each phosphatidylethanolamine, measured by steady-state fluorescence anisotropy of *cis*-parinaric acid, was lower at pH 10 than at pH 8 or in water. The ability of dimethylsuberimidate to discriminate between phosphatidylethanolamines in the fluid and gel phases should allow use of this reagent to identify phosphatidylethanolamine species within the gel or fluid lipid phase.

Introduction

There is strong evidence that, in both model (see for example, Refs. 1–5) and biological (see for example, Refs. 6–10) membranes, lipid domains exist. Such domains may represent different lipid phases [1,2,6–10] or lateral groupings of lipids within a phase [3–5]. For mixtures of two or more lipids in the same phase or for mixtures of more than two lipids in two phases, there is presently a paucity of techniques capable of determining the compositions of these domains. Thus we have proceeded to develop a method of determining nearest neighbor PE species by chemical cross-linking, planning to extrapolate data on nearest neighbors to yield information about lipid domain composition.

The cross-linking reagent dimethylsuberimidate has been reported to permeate membranes and react with the free amino groups of fluid phase phosphatidylethanolamines in aqueous dispersions, producing dimers with amidine linkages [11,12]. The production of PE dimers is favored at higher pH, between pH 7 and 10. Mixtures of dimeric species can be resolved into individual components by chromatographic techniques, including C_{18} reversed phase HPLC.

In this report we demonstrate that dimethylsuberimidate cross-linking is sensitive to the phase of the PE. Under the conditions used in these studies, fluid phase PEs are cross-linked more readily than gel phase PEs. Indeed, the gel-to-fluid phase transition can be detected by quantitating dimethylsuberimidate cross-linking as a function of temperature. Thus reaction of PE with dimethylsuberimidate can potentially be used as a marker for those species which are in the fluid phase in a mixture of gel and fluid lipid phases.

Materials and Methods

Materials. PEs were purchased from Avanti Polar Lipids, Inc., Birmingham, AL or Sigma Chemical Co., St. Louis, MO. Dimethylsuberimidate dihydrochloride was from Pierce Chemical Co., Rockford, IL. Silica gel G thin-layer plates (20 × 20 cm) or high-efficiency, prescored silica gel plates (10 × 20 cm) with inorganic binder were from Analtech, Inc., Newark, DE. Hexane and water used for HPLC were HPLC grade from Fisher Scientific, St. Louis, MO. Ethanol for HPLC was from Midwest Solvents Co. of Illinois, Pekin, IL and was filtered through a 0.45 μm filter or redistilled prior

Abbreviations: PE, phosphatidylethanolamine; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; cPnA, *cis*-parinaric acid (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid); DPPE, dipalmitoylphosphatidylethanolamine (dihexadecanoyl PE); DMPE, dimyristoylphosphatidylethanolamine (ditetradecanoyl PE); DLPE, dilauroylphosphatidylethanolamine (didodecanoyl PE); DEPE, dielaidoylphosphatidylethanolamine (di-9-*trans*-octadecaenoyl PE); DOPE, dioleoylphosphatidylethanolamine (di-9-*cis*-octadecaenoyl PE).

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to use. All other reagents were from Sigma, Fisher, or Aldrich Chemical Co., Milwaukee, WI.

Vortexed lipid vesicles. Phospholipid concentration was determined by phosphate assay [13]. A measured quantity of each PE dissolved in solvent was placed under a nitrogen stream and dried further under vacuum. Buffer was added and the PEs were allowed to hydrate over a several hour period. Each sample was incubated at a temperature at least 10°C above the gel-to-fluid phase transition temperature of the PE and then vigorously vortexed. Buffers used to maintain reaction pH were 0.1 M NaCl, 0.05 M sodium borate (pH 8.0) and 0.1 M NaCl, 0.05 M sodium bicarbonate (pH 10.0). Vesicles made by this method (pH 10) were sealed as indicated by their ability to trap radiolabelled glucosamine [12].

Sonicated lipid vesicles. 1 mM PE in vortexed vesicles was sonicated to optical clarity above the phase transition temperature of the lipid with a probe-type sonicator.

Steady-state fluorescence anisotropy measurements. 400 nmol PE in vortexed lipid vesicles in 4 ml buffer were placed in a quartz cuvette in the thermostatted, temperature-controlled cuvette chamber of a Spex 1902 Fluorolog spectrofluorometer. This instrument was equipped with quartz polarizers obtained from Spex (Edison, NJ). The limiting fluorescence anisotropy (for fluorescein in alkaline glycerol at 10°C [14]) was 0.35 for this instrument. The samples were excited at 325 nm and the excitation bandpass was 5.0 nm. Fluorescence emission was monitored at 420 nm with a 20.0 nm bandpass. 4 nmol cPnA in 8 μl ethanol, containing 1 mol butylated hydroxytoluene/10 mol cPnA, was injected into the vesicle suspension and mixed thoroughly. Fluorescence emission parallel and perpendicular to the vertically polarized excitation light was monitored as the stirred sample was cooled at a rate of 0.5 C°/min, controlled by a circulating water bath and a linear temperature programmer. Data obtained while the samples were heated (not shown) were equivalent to those obtained while the samples were cooled. The temperature corresponding to the midpoint of the transition was taken as the temperature halfway between the temperature at which the anisotropy began to increase and the temperature at which the increase ended.

Cross-linking reaction. PE vesicles were equilibrated in the appropriate buffer at the indicated temperature. The PE vesicles were mixed. 1 mol dimethylsuberimidate dihydrochloride/2 mol PE then was dissolved in a small volume of identical buffer with the addition of two moles sodium hydroxide per mole dimethylsuberimidate dichydrochloride. Immediately after dissolving the dimethylsuberimidate and 1 min after combining the different PE vesicles, an aliquot of the dimethylsuberimidate solution was added to the vesicle mixture to begin the reaction. The final PE concentration in the reaction mixture was 0.5 mM. The reaction was terminated by extraction with chloroform and methanol. The water phase was re-extracted twice with chloroform and the combined organic phase was evaporated under nitrogen. Dimeric PEs generated in this way then were analyzed either by HPLC directly or, for more complex mixtures containing PEs of widely varying chain length, by HPLC after a separation from non-dimeric species by TLC. For TLC analysis, the material was redissolved in chloroform/methanol (2:1, v/v) and used directly. For HPLC analysis, the material was dissolved in a larger volume of chloroform/methanol (2:1, v/v) and prepared as described by Roth et al. [12].

Thin-layer chromatography. Two-dimensional thin-layer chromatography on silica gel G plates with inorganic binder was performed and the 'major reaction products' were isolated from the plates as previously described [12]. The plates were developed with chloroform/methanol/acetic acid (65:25:8, v/v/v) in the first dimension and chloroform/methanol/water (65:25:4, v/v/v) in the second dimension.

High-performance liquid chromatography. Dimeric phosphatidylethanolamine species were chromatographed on a 4.6 × 250 mm Altex Ultrasphere ODS column (C_{18} reversed phase) (Beckman Instruments, Inc., San Ramon, CA). Mixtures of dimers were eluted isocratically or by a program which included a linear gradient. The solvent for isocratic elution was 20 mM choline chloride in ethanol/water/hexane (77:13:10, v/v/v). The gradient program began with an isocratic elution with 20 mM choline chloride in ethanol/water/hexane (78:16:6, v/v/v) for 15 min. Elution continued for 1 h with a linear gradient of 20 mM choline chloride in ethanol/water/hexane (78:16:6, v/v/v) and increasing amounts of 20 mM choline chloride in ethanol/water/hexane (79:11:10, v/v/v) up to 100% 20 mM choline chloride in ethanol/water/hexane (79:11:10, v/v/v). Elution then continued isocratically with the second solvent mixture. In all cases the flow rate was 1.0 ml/min. Detection was by absorbance at 205 nm. At this wavelength, the absorption coefficients of dimers of various PE molecular species are similar [12]. The identity of the amidine dimers, in all cases, was determined by analytical methods as previously described [12]. Dimers were quantitated by weighing the sample peaks and applying a conversion factor between sample peak weights and the peak weight obtained by chromatographing a known amount of dimeric material.

Results

The gel-to-fluid phase transition of DPPE in pH 10 buffer, pH 8 buffer, and water, as detected by steady-state fluorescence anisotropy of cPnA is shown in Fig. 1. The midpoint temperatures of the gel-to-fluid phase

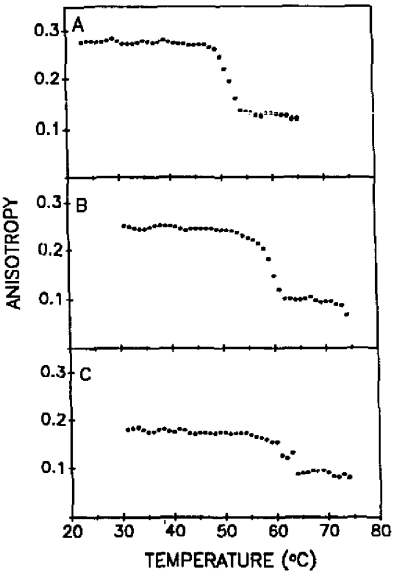

Fig. 1. Steady-state fluorescence anisotropy of cPnA in DPPE in (A) pH 10 buffer, (B) pH 8 buffer, and (C) water.

transitions of DPPE, DMPE, DEPE and DLPE, obtained by this method are shown in Table I. The transition temperatures obtained for each of the lipids dispersed in water are similar to some of the lower values previously published. The pK of PE is roughly 10, and the gel-to-fluid transition temperature of each PE decreased with increasing pH as the amine group was deprotonated. The transitions also were broadened at pH 10 as compared to the transitions in water.

TABLE I

Gel-to-fluid phase transition temperatures as determined by steady-state fluorescence anisotropy of cPnA

Lipid	T_m (°C) [a]			T_c in H_2O from literature	
	at pH 10 [b]	at pH 8 [c]	in H_2O	(°C)	(°C)
DPPE	52.0	60.0	62.0	60.8 [d]	63.5 [e]
DMPE	37.0	46.0	47.0	47.5 [d]	49.5 [f]
DEPE	23.0	31.5	35.0	35.0 [d]	38.5 [g]
DLPE	20.0	27.0	29.0	29.0 [d]	
DOPE	n.d. [h]	n.d.	n.d.	−16.0 [d]	−16.0 [i]

[a] T_m represents the midpoint of the transition as determined by fluorescence anisotropy of cPnA, as described in Materials and Methods.
[b] The buffer was 0.1 M NaCl, 0.05 M sodium bicarbonate (pH 10).
[c] The buffer was 0.1 M NaCl, 0.05 M sodium borate (pH 8).
[d] DSC measurements [15].
[e] DSC measurements [16].
[f] DSC measurements [17].
[g] DSC measurements [18].
[h] Not determined.
[i] DSC measurements [19].

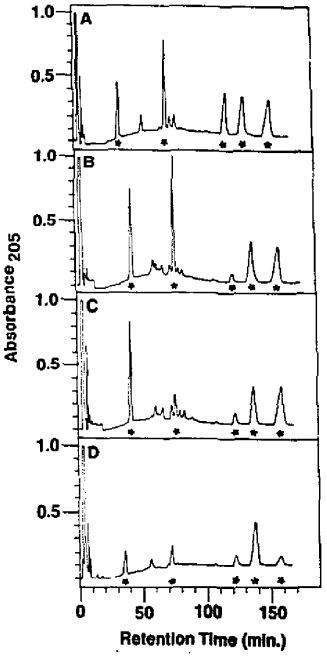

Fig. 2. HPLC separation of dimers formed from reactions (pH 10) of 1.25 μmol dimethylsuberimidate with a mixture of vortexed vesicles of 0.5 μmol DLPE, vortexed vesicles of 0.5 μmol DMPE, vortexed vesicles of 0.5 μmol DPPE, vortexed vesicles of 0.5 μmol DOPE, and vortexed vesicles of 0.5 μmol DEPE. The reaction mixtures were extracted, subjected to TLC, and the major reaction products isolated as described in Materials and Methods. These products then were subjected to C_{18} reverse phase HPLC, using the gradient program. The asterisks indicate the position, in order of retention time, of DLPE homodimer, DMPE homodimer, DPPE homodimer, DOPE homodimer, DEPE homodimer. The reactions were performed at (A) 70 °C for 0.5 h, (B) 50 °C for 2 h, (C) 30 °C for 8 h, and (D) 10 °C for 32 h. The peaks at 70 °C represent 88 nmol DLPE homodimer, 137 nmol DMPE homodimer, 113 nmol DPPE homodimer, 126 nmol DOPE homodimer, and 141 nmol DEPE homodimer, or an overall reaction yield of 24%. At 50 °C, the peaks represent 138 nmol, 148 nmol, 19 nmol, 156 nmol, and 146 nmol of each homodimer, respectively, or an overall reaction yield of 24%. At 30 °C, the peaks represent 149 nmol, 34 nmol, 30 nmol, 131 nmol, and 181 nmol of each homodimer, respectively, or an overall reaction yield of 21%. At 10 °C, the peaks represent 46 nmol, 37 nmol, 29 nmol, 155 nmol, and 33 nmol of each homodimer, respectively, or an overall reaction yield of 12%.

The formation of dimethylsuberimidate dimers as a function of the phase of the PE was examined. In the experiment described in Figs. 2 and 3, equimolar amounts of DPPE, DMPE, DEPE, DLPE, and DOPE were dispersed separately into pH 10 buffer, then mixed and cross-linked at various temperatures. After purification of the dimers as a group by TLC, the individual species were separated by HPLC as shown in Fig. 2. As can be seen in this figure, as 70 °C, above the phase transition at pH 10 of any of these PE species, approximately equal amounts of each of the five dimeric

species are formed. At 50°C, below the transition of DPPE at pH 10 (52°C by cPnA fluorescence anisotropy measurements), there was a reduced amount of DPPE dimer, as compared to the amount formed at 70°C and as compared to the amount of the other four dimeric species at 50°C. At 30°C, below the transition of DMPE (detected at 37.5°C by fluorescence at pH 10), the amount of DMPE dimer, as well as DPPE dimer, was lower than the amount of the three other dimers. At 10°C, below the transitions at pH 10 of DEPE at 23.5°C and DLPE at 20.5°C, the amount of DOPE dimer formed was considerably greater than the amount of dimer formed by any of the other species. The transition of DOPE from gel to fluid at pH 10 undoubtedly occurs below 0°C.

It should be noted that no dimers are formed between PEs in different vesicles, indicating that the PE vesicles remain discrete throughout the reaction. Such heterodimers would elute from the HPLC column between the homodimers shown in Fig. 2 [12]. For example, a dimer of DPPE and DOPE would elute between the DPPE homodimer and the DOPE homodimer.

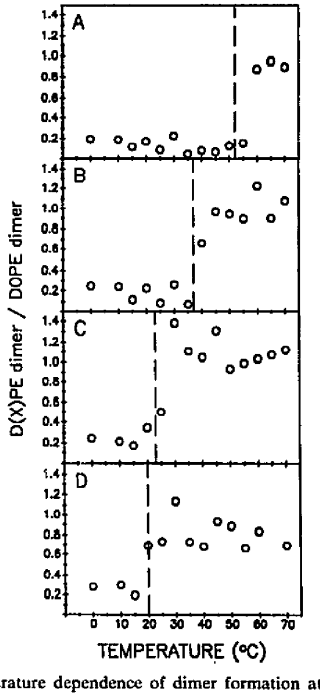

Fig. 3. Temperature dependence of dimer formation at pH 10. The cross-linking reaction and separation of the dimeric products were performed as described in the legend to Fig. 2. The reaction times were 0.5 h at 70°C, 1 h at 65°C and 60°C, 2 h at 55°C and 50°C, 4 h at 45°C and 40°C, 8 h at 35°C and 30°C, 16 h at 25°C and 20°C, 32 h at 15°C and 10°C, and 64 h at 0°C. The amounts of (A) DPPE dimer, (B) DMPE dimer, (C) DEPE dimer, and (D) DLPE dimer are normalized to the amount of DOPE dimer formed at each temperature. The dashed lines indicate the temperature of the gel-to-fluid phase transition temperature at pH 10 as detected by steady-state fluorescence anisotropy of cPnA.

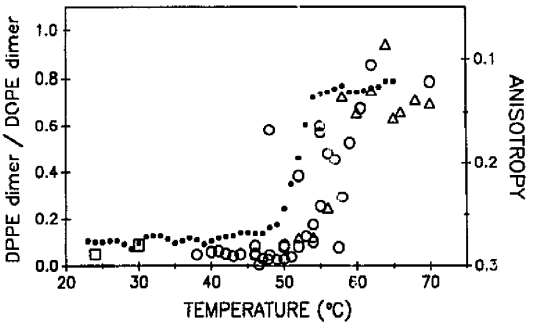

Fig. 4. Temperature dependence of DPPE dimer formation and cPnA steady-state fluorescence anisotropy at pH 10. Dimers were formed from reactions (pH 10) of 0.5 μmol dimethylsuberimidate with a mixture of vortexed vesicles of 0.5 μmol DPPE and vortexed vesicles of 0.5 μmol DOPE. The reaction mixtures were extracted and applied directly to a C_{18} reverse phase HPLC column and eluted with the isocratic solvent system. The amount of DPPE dimer at each temperature is normalized ot the amount of DOPE dimer formed at that temperature. Triangles represent cross-linking reactions which were carried out for 2 h, circles represent reactions which were carried out for 3 h, and squares represent reactions which were carried out for 6 h. The dots are the steady-state fluorescence anisotropy data shown in Fig. 1A (shown here with the anisotropy scale inverted).

Fig. 3 shows the amount of dimeric products from the reaction of equimolar amounts of DPPE, DMPE, DEPE, DLPE, and DOPE in separate vesicles at pH 10 with dimethylsuberimidate at temperatures from 0 to 70°C. The amounts of DPPE dimers, DMPE dimers, DEPE dimers, and DLPE dimers are normalized to the amount of DOPE dimer formed. Although the amount of DOPE dimer formed was similar at each temperature (Fig. 2), use of the DOPE dimer as an 'internal standard' corrected for any variations in the recovery of dimers during the extraction and TLC steps. For each PE species, a marked change in the amount of dimers formed occurred at or very close to the temperature of the gel-to-fluid phase transitions.

Fig. 4 shows the amount of DPPE dimer formation as compared to DOPE dimer formation as the DPPE undergoes its gel-to-fluid transition. In this case, the midpoint of the transition, as detected by the ability to form dimers, was about 3 to 4 C° higher than the transition detected by cPnA fluorescence anisotropy. However, as pointed above, the transition temperatures detected by cPnA fluorescence anisotropy were lower than those obtained by some other workers.

In order to determine whether the differences in the amount of dimer formed between the two phases might be specific to vortexed vesicles, the experiment shown in Fig. 5 was performed. Sonicated vesicles of DPPE, DOPE, and DEPE at pH 10 were reacted with dimethylsuberimidate at 70°C, 40°C, and 10°C. The results

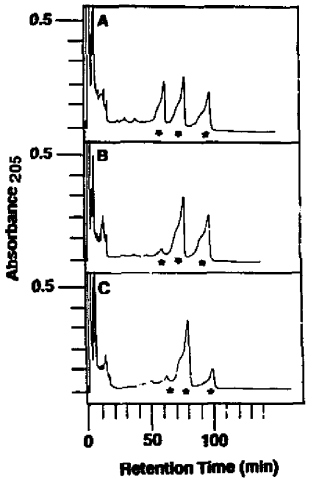

Fig. 5. HPLC separation of dimers formed from reactions at pH 10 of 2.25 μmol dimethylsuberimidate with a mixture of sonicated vesicles of 1.5 μmol DPPE, 1.5 μmol DOPE, and 1.5 μmol DEPE. The reaction mixtures were extracted, and one third of each sample was applied directly to a C_{18} reverse phase HPLC column and eluted with the isocratic solvent system. The asterisks indicate the position, in order of retention time, of DPPE homodimer, DOPE homodimer, and DEPE homodimer. The reactions were performed at (A) 70 °C for 0.5 h, (B) 40 °C for 4 h, and (C) 10 °C for 32 h. The peaks at 70 °C represent 112 nmol DPPE homodimer, 150 nmol DOPE homodimer, and 159 nmol DEPE homodimer, or an overall reaction yield of 28%. At 40 °C, the peaks represent 23 nmol, 197 nmol, and 196 nmol of each homodimer, respectively, or an overall reaction yield of 28%. At 10 °C, the peaks represent 13 nmol, 235 nmol, and 73 nmol of each homodimer, respectively, or an overall reaction yield of 21%.

shown in Fig. 5 are similar to those shown in Fig. 2, indicating that the sensitivity of dimethylsuberimidate to lipid phase was not dependent on the method of lipid dispersion. In an additional experiment, the amount of cross-linking in gel phase vesicles made by sonicating or vortexing was compared directly by cross-linking at 0 °C for 128 h at pH 10. DMPE (0.5 μmol) produced 26 ± 3 nmol dimer when dispersed in vortexed vesicles and 29 ± 1 nmol dimer when dispersed by sonication. DPPE (0.5 μmol) produced 21 ± 4 nmol dimer when dispersed in vortexed vesicles and 28 ± 6 nmol dimer when dispersed by sonication.

Figs. 6 and 7 show the amount of dimer formed in vortexed vesicles as a function of temperature between 0 °C and 70 °C at pH 8, rather than at pH 10 as shown in the previous figures. In this experiment, DOPE was excluded because it did not form vesicles easily at pH 8. Instead, the absolute amount of each dimer is reported. Fewer dimers were formed at pH 8 than at pH 10, as reported previously [12]. There also were several reaction side-products formed at pH 8 but not at pH 10 (Fig. 6). The peak immediately preceding the DMPE dimer peak was a derivative of DLPE while the side product chromatographing between the DPPE dimer

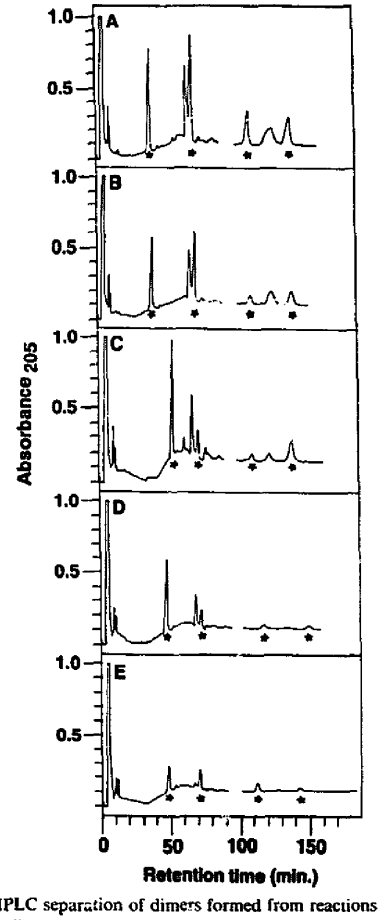

Fig. 6. HPLC separation of dimers formed from reactions at pH 8 of 3.0 μmol dimethylsuberimidate with a mixture of vortexed vesicles of 1.5 μmol DLPE, vortexed vesicles of 1.5 μmol DMPE, vortexed vesicles of 1.5 μmol DPPE, and vortexed vesicles of 1.5 μmol DEPE. The reaction mixtures were extracted, subjected to TLC, and the major reaction products isolated as described in Materials and Methods. The products then were subjected to C_{18} reverse phae HPLC, using the gradient program. The asterisks indicate the position, in order of retention time, of DLPE homodimer, DMPE homodimer, DPPE homodimer, and DEPE homodimer. The reactions were performed at (A) 65 °C for 2 h, (B) 50 °C for 4 h, (C) 40 °C for 8 h, (D) 25 °C for 32 h, and (E) 0 °C for 128 h. (A short section of each chromatogram is omitted. A compound which we had attempted to use as an internal standard eluted in this area. The use of this compound as an internal standard was unsuccessful because there were other small peaks eluting at similar times.) The peaks at 65 °C represent 134 nmol DLPE homodimer, 148 nmol DMPE homodimer, 88 nmol of DPPE homodimer, and 94 nmol of DEPE homodimer, or an overall reaction yield of 7.7%. At 50 °C, the peaks represent 89 nmol, 97 nmol, 24 nmol, and 45 nmol of each homodimer, respectively, or an overall yield of 4.3%. At 40 °C the peaks represent 112 nmol, 39 nmol, 17 nmol, and 62 nmol of each homodimer, respectively, or an overall yield of 3.8%. At 25 °C, the peaks represent 105 nmol, 26 nmol, 12 nmol, and 12 nmol of each homodimer, respectively, or an overall yield of 2.6%. At 0 °C, the peaks represent 32 nmol, 25 nmol, 18 nmol, and 10 nmol of each homodimer, respectively, or an overall yield of 1.4%.

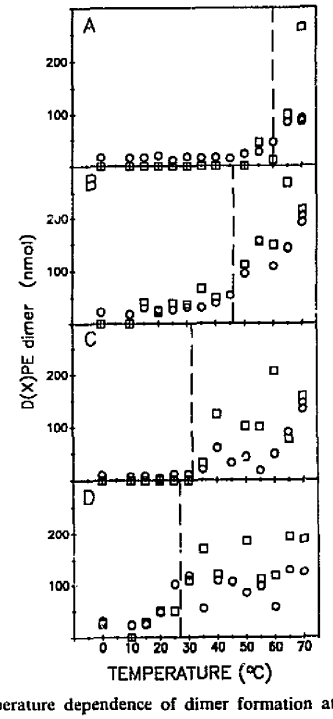

Fig. 7. Temperature dependence of dimer formation at pH 8. The cross-linking reaction and separation of the dimeric products were performed as described in the legend to Fig. 6. The reaction times were 1 h at 70 °C, 2 h at 65 °C and 60 °C, 4 h at 55 °C and 50 °C, 8 h at 45 °C and 40 °C, 16 h at 35 °C and 30 °C, 32 h at 25 °C and 20 °C, 64 h at 15 °C and 10 °C, and 128 h at 0 °C. The panels represent the amount of (A) DPPE dimer, (B) DMPE dimer, (C) DEPE dimer, and (D) DLPE dimer. In the data set represented by the circles, the entire sample was analyzed by HPLC. In the data set represented by the squares, one third of the sample was analyzed by HPLC and the peak areas were multiplied by three. The dashed lines indicate the temperature of the gel-to-fluid phase transition temperature at pH 8 as detected by steady state fluorescence anisotropy of cPnA.

and the DEPE dimer was derived mostly from DMPE. Still, the phase sensitivity of the dimethylsuberimidate cross-linking reaction was similar to that seen at pH 10. There was a reduced amount of each dimer formed at temperatures below the gel-to-fluid phase transition temperature of each PE as compared to the amount of dimer formed at temperatures above the gel-to-fluid phase transition (Fig. 7).

Discussion

Our main objective in developing a method of cross-linking lipids is to determine the arrangement of the various phosphatidylethanolamine species within the *Escherichia coli* inner membrane. Since these membranes are composed largely of PE, most of the diversity in the phospholipids is in the acyl chains. Cross-linking should accurately identify the nearest neighbor species within these membranes, as well as identify the lipid species in each phase in the membranes in *E. coli* grown under conditions which promote the existence of gel phase lipid [20,21]. We plan to use cross-linking in combination with measurements of the steady-state fluorescence anisotropy of various parinaroyl phosphatidylethanolamine and phosphatidylglycerol probes to derive a detailed picture of the lipid organization of *E. coli* membranes with a variety of lipid phase and molecular species compositions [20,22,23].

The present study used model membranes to demonstrate that the reaction of dimethylsuberimidate with PE, resulting in the formation of dimers between molecules within the same vortexed or sonicated vesicles, is sensitive to the phase of the lipid. About four times more dimers were formed between fluid phase PEs than between gel phase PEs. It was demonstrated that this cross-linking reaction can be performed at both pH 8 and at pH 10. Although some additional side products were formed at pH 8 as compared to pH 10 in a mixture of DLPE, DMPE, DPPE, and DEPE, the products which interfered with dimer analysis were derived from DLPE and DMPE. No interfering products are observed at pH 8 in a mixture of the more common biological PE species, those containing 16 and 18 carbon acyl groups (Roth, M.R. and Welti, R., unpublished data). Taken together, these data suggest that dimethylsuberimidate cross-linking should be applicable to biological membranes.

An additional finding was that the gel-to-fluid transition temperature of each PE was decreased as the pH was increased to pH 10. Other workers have observed this phenomenon and have attributed it to the loss of hydrogen bonding between the $-NH_3^+$ groups and the $-PO_4^-$ groups on deprotonation of the $-NH_3^+$ groups [24]. This loss of hydrogen bonding at high pH expands the area per PE head group, thus favoring the liquid-crystalline as compared to the gel or the inverted hexagonal phases, in which PEs have reduced head group areas [24]. Indeed, no hexagonal phase structures were observed at pH 9.2 in vesicles made from DMPE or *E. coli* PE [25].

The design of this study was such that the time allowed for dimer formation was longer as the reaction temperature was decreased. This protocol allowed for complete degradation (presumably hydrolysis [11]) of unreacted dimethylsuberimidate before extraction, preventing additional cross-linking during the extraction procedure.

The basis for the greater yield of dimer in the fluid, as compared to the gel phase is unclear and deserved further investigation. One possibility is that dimethylsuberimidate penetrates fluid phase vesicles more

quickly than it penetrates gel phase vesicles. The similarity of the results obtained using sonicated (presumably single bilayer) and vortexed (presumably multilamellar) vesicles would argue against this interpretation, unless the vesicles underwent morphological change at and/or below the phase transition temperature. An alternative explanation for the greater yield of dimers in the fluid phase is that the reaction of dimethylsuberimidate with fluid phase PE competes more favorably with the hydrolysis of dimethylsuberimidate than does the reaction with gel phase PE. It can be hypothesized that this is due to the distance between and conformation of the amino groups in the two phases in relation to the length of the dimethylsuberimidate molecule. The distance between the PE nitrogens in the amidine dimer formed with dimethylsuberimidate is about 1.1 nm. The average dimater of a PE molecule in gel phase is about 0.7 nm, while the average diameter of PE in a liquid-crystalline lamellar phase is at least 0.8 nm [26]. The greater distance between the amine groups, along with the less rigid packing of the fluid phase, may make it easier for the fluid phase to accommodate the formation of dimethylsuberimidate cross-links.

A question arises in the study of nearest neighbor lipids by cross-linking as to the rate of cross-linking as compared to the rate of lipid diffusion. It has been estimated that the time it takes for a lipid molecule to move from one end of a bacterium to the other is 1 s [27]. Thus, in order to cross-link nearest neighbor PEs, one of two conditions must hold: either (1) the cross-linking reaction must be very fast so that cross-linking occurs before PE movement, or (2) the PE which has reacted with dimethylsuberimidate and has not yet reacted with a second PE molecule, must have nearest neighbor relationships similar to an unreacted PE molecule with the same acyl chains. While it is difficult to demonstrate that the first condition holds, we believe that it is reasonable to speculate that the second condition holds in a mixture of lipid species such as those found in *E. coli*, in which most of the phospholipid diversity is in the acyl chains. Previous work has shown that in PE/phosphatidylcholine mixtures, partitioning of lipids into domains depends on lipid acyl chains and transition temperatures, rather than on their head groups [28]. In order to assess the degree of perturbation of the PE phase properties which might be expected from the half-reaction with dimethylsuberimidate, as an analogue of the half-reacted compound, we prepared PE reacted with the mono-reactive reagent, ethylacetimidate. The T_m of the prepared compound (pH 10) was 48.5°C, as compared to 52°C for unreacted PE (Avery, R.B. and Welti, R., unpublished data). This suggests that the half reaction of dimethylsuberimidate with PE is unlikely to have a large effect on PE nearest neighbor relationships. Still, this point deserves further investigation.

There have been a number of previous studies in which lipids were cross-linked using amine-specific reagents. While Gaestel et al. [29] employed model membranes in their dinitridifluorobenzene cross-linking studies, most of the studies have been performed on biological lipid mixtures (see, for example, Refs. 30–33). These workers have attempted to draw conclusions about the arrangement of the amino lipids from the cross-linking patterns. While most of the biological lipids examined are probably in a fluid phase, in the absence of careful studies on model systems it is difficult to ascertain whether non-random cross-linking patterns are due to membrane lipid arrangement or to properties of the cross-linking reagents.

A careful study describing the properties of cross-linking by photoactivatable carbene-generating phospholipids in model membranes was performed by Curatolo et al. [34]. The photoactivatable lipids form dimers with phosphatidylcholines in the fluid phase preferentially; the amount of fluid phase cross-linking is roughly three to eight times greater than the amount of solid phase cross-linking. This degree of preference for fluid phase cross-linking is similar to that reported here. While dimethylsuberimidate cross-linking is limited to amino lipids, the photoactivatable reagents have the advantage of being able to react with any membrane lipid. This property might allow use of these lipids to identify the components of the fluid phase in a complex biological mixture of lipids with multiple head groups. In practice, however, there are several difficulties with the use of these reagents. One problem is that the photoactivatable lipids form cross-links with particular species within the fluid phase more readily than with others. Dimethylsuberimidate, in contrast, has similar reactivity with various fluid phase species. A more serious problem with the photoactivatable lipids is the number of products formed between these reagents and a single membrane lipid [35]. To get around the analytical problem which this property poses, these workers used model lipid mixtures in which only one species was radiolabelled, then quantitated the radiolabel in the cross-linked pool [34]. It would be tedious at best to examine a biological mixture which included one or two labelled species at a time. With dimethylsuberimidate cross-linking of PEs from *E. coli* strains with limited molecular species heterogeneity, analysis of virtually all the nearest neighbor relationships is certainly feasible.

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