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Arrangement of phosphatidylethanolamine molecular species in *Escherichia coli* membranes and reconstituted lipids as determined by dimethyl suberimidate cross-linking of nearest neighbor lipids

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Abstract

Dimethylsuberimidate cross-linking has been used to determine the arrangement of phosphatidylethanolamine (PE) molecular species in *Escherichia coli* membranes. No large deviations from random mixing were found in wild-type strain AB1623, either in whole cells or in extracted lipids which were reconstituted into multilamellar vesicles. These results suggest, first, that there is little difference in the PE molecular species composition of the three lipid monolayers (the inner and outer monolayers of the inner membrane and the inner monolayer of the outer membrane) which contain significant amounts of PE. Secondly, the results suggest that the molecular species within each monolayer and in the extracted lipids are arranged close to randomly with no tendency for like molecular species to cluster. *E. coli* strain L8-2, which has a defect in β-oxidation and a temperature-sensitive mutation in total fatty acid synthesis, was grown on *cis*-vaccenate (*cis*-11,12-octadecenate) to enrich the cells in divaccenoyl PE. Again, in whole cells or in lipids extracted from whole cells and reconstituted into multilamellar vesicles, the species were close to randomly arranged. However, a consistent, slight tendency of divaccenoyl species to pair with like species as compared to pairing with the second most common species, vaccenoyl, palmitoleoyl PE, was noted in both extracted lipids and in whole cells.

Key words: Bacterium; Phospholipid; Phosphatidylethanolamine; Crosslinking; Domain; Fluid–fluid immiscibility

1. Introduction

A domain of lipids is defined as any lateral grouping with a composition which differs from other lateral groupings within the same bilayer. It seems clear that an understanding of the arrangements of membrane components and the chemical and physical basis for these arrangements may have wide application to understanding membrane function. An ultimate understanding of the arrangement of membrane components must include an understanding of the arrangement of lipid molecular species.

Recently, Rodgers and Glaser [1] have used fluorescence microscopy to detect non-homogeneous arrangements of added fluorescent phospholipids in erythrocytes. These workers determined that fluorescent phosphatidylcholines, for example, seem to be enriched in one area of the membrane while fluorescent phospholipids with other head groups are clustered in other areas. This exciting result certainly lends strong support to the concept of domains in biological membranes. Still, the compositions of the domains detected by this method are difficult to assess in a quantitative manner and, because the fluors are attached to the acyl chains of the phospholipids, these workers have focused on studying the role of head groups, rather than acyl chains, in domain formation.

De Bony and coworkers [2] used photodimerization of anthracene to investigate the transverse and lateral distribution of lipids with different head groups in the Gram-positive bacterium *Micrococcus luteus*. By comparing the cross-linking patterns they obtained with those predicted for random cross-linking, they deter-

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Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin or diphosphatidylglycerol; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; DMS, dimethyl suberimidate; 17 cp, dihydrosterulic acid or 8-[2-octyl*cyclo*propanyl]octanoic acid, a fatty acid with a total of 17 carbons, including a cyclopropane ring at the 9,10 position.

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mined that the two major lipids, phosphatidylglycerol and dimannosyldiacylglycerol, were not homogeneously distributed in the plane of the bacterial membrane. This is perhaps the most quantitative picture of the arrangement of lipid species in a biological membrane thus far obtained. Again, these workers focused on the arrangement of different head group classes of membrane lipids.

We have investigated the arrangement of molecular species of phospholipids in the membranes of *Escherichia coli*. The *E. coli* membrane phospholipids are composed of about 78% PE, 15% PG and 7% CL. *E. coli* membranes were chosen for this investigation for several reasons: (1) because of the high content of PE, a phospholipid which is capable of reacting specifically with DMS, yielding dimeric products which can be readily analyzed [3], (2) because the mixture of *E. coli* membrane lipid molecular species is not as complex as that of most eukaryotic cells, with most of the *E. coli* heterogeneity among the PE species, allowing us to examine the role of acyl chains in membrane lipid arrangement, and (3) because of the availability of a mutant *E. coli* strain, L8-2, which has a defect in β-oxidation and a temperature-sensitive mutation in total fatty acid synthesis [4]. By supplementing this strain with *cis*-vaccenate (*cis*-11,12-octadecenate), the molecular species composition can be simplified with divaccenoyl phospholipids becoming the predominant species [5]. TEMPO partition measurements on lipids derived from strain L8-2 grown with vaccenate showed a break at 46°C, which was postulated to correspond to the breakup of quasi-crystalline arrays of divaccenoyl lipids in the liquid crystalline phase [5]. These results suggested that detection of phospholipid clustering in the fluid membrane of this strain might be possible and thus served as an impetus for our investigation. DMS forms dimers of PEs in the fluid phase more readily than dimers of PEs in the gel phase, but does not show any preference for reaction with PEs of any particular molecular species within a phase [6]. It penetrates fluid phase lipid bilayers quickly [7]. We have previously used this reagent to investigate the miscibility of mixtures of fluid-and gel-phase synthetic PEs [8,9]. Our previous results demonstrated that DMS cross-linking of PEs allows detection of lateral immiscibility; at the same time, it is unlikely that the reagent induces artifactual domain formation, since, in the fluid-phase model systems studied, the PE molecular species were found to be randomly or nearly randomly arranged.

2. Materials and methods

2.1. Escherichia coli strains

Strains AB1623 and L8-2 are *E. coli* K12 derivatives. Strain AB1623 has normal fatty acid and glycerolipid synthesis [10]. Strain L8-2 is a derivative of strain AB1623 which has a β-oxidation defect (*fad*E), which was introduced by conjugation, and a temperature-sensitive mutation affecting total fatty acid biosynthesis (*fab*E) [4]. These strains have been generously provided by Dr. David F. Silbert of Washington University Medical School.

2.2. Bacterial growth

Minimal growth medium for these strains was Medium 63 [11] containing 0.4% glycerol, 5 mM potassium glutamate, 1 μg thiamine/ml, and 0.5 μg yeast extract/ml. Strain AB1623 was grown at 37°C without other supplement. Strain L8-2 was grown at 30°C without other supplement and subcultured at 36.7°C in media with fatty acid supplement. *cis*-Vaccenate solution (100 mg/ml) was prepared by dissolving 1 g of vaccenic acid in 8 ml ethanol, adding 352 μl 10 M KOH, and bringing the volume to 10 ml with water. To prepare the supplemented media, 1 ml of *cis*-vaccenate was added to each liter of the minimal growth media containing 0.1% Brij-58 (to solubilize the vaccenate supplement) to give a final fatty acid concentration of 100 μg/ml.

2.3. Harvesting bacteria

Bacteria were harvested in log phase growth when the A_{600} was 0.7–1.0. Bacteria were centrifuged at 4°C for 10 min at $4100 \times g$. Strain AB1623 bacteria were washed twice with Medium 63 and those of strain L8-2 were washed three times in Medium 63 with 0.1% Brij. The bacteria were resuspended in 100 mM NaCl, 50 mM KH_2PO_4 (pH 8), centrifuged, and the pellet resuspended a second time in the same buffer.

2.4. Cross-linking of whole cells for dimer analysis

The PE concentration of the culture was estimated by assuming that 1·1 of cells at an A_{600} of 1.0 would have 24 μmol of PE. Based on this, 0.5 mol DMS was used per mol PE in the whole cell cross-linking reactions used for molecular species analysis. Actual DMS/PE ratios were 0.4 to 0.5, as determined by later analysis of PE content. Dimethylsuberimidate dihydrochloride (with 2 mol NaOH added per mol dimethyl suberimidate dihydrochloride) in 100 mM NaCl, 50 mM KH_2PO_4 (pH 8) was reacted with the whole cells for 10 min at 37°C. Bacteria containing between 10 and 20 μmol of PE were utilized in each cross-linking reaction (corresponding to one HPLC run). Reactions volume were 5–8 ml. Reactions were stopped by adding glacial acetic acid to decrease the pH to 4.

2.5. Isolation of phospholipids and formation of multilamellar vesicles

E. coli lipids were extracted from the resuspended cells by the procedure of Bligh and Dyer [12]. The solvent was evaporated under vacuum in a rotary evaporator, and the lipids were redissolved in chloroform. The lipids were applied to an activated silicic acid column which was washed with chloroform. The phospholipids were eluted with methanol and the solvent was evaporated. The lipids were dissolved in a known volume of chloroform/methanol (2:1, v/v). The phospholipids were quantitated and the head group composition was determined as described under 'Phospholipid analysis'. Multilamellar lipid vesicles were prepared by removing chloroform/methanol from isolated bacterial lipid by nitrogen evaporation, followed by placing the sample in a vacuum. Buffer (100 mM NaCl, 50 mM KH_2PO_4, pH 8) at 37°C was added to the lipid film to make the PE concentration 1–2 mM. A glass bead was added, and the tube was vortexed until the lipid was dispersed.

2.6. Cross-linking of isolated phospholipids

E. coli total phospholipids in vortexed vesicles were reacted with dimethyl suberimidate dihydrochloride (with 2 mol NaOH added per mol dimethyl suberimidate dihydrochloride) in 100 mM NaCl, 50 mM KH_2PO_4 (pH 8) for 10 min at 37°C. The ratio of DMS to PE was always between 0.25 and 0.5. Between 5 and 10 μmol of PE were utilized per cross-linking reaction (corresponding to one HPLC run). The reaction was stopped by adding glacial acetic acid to decrease the pH to 4.

2.7. Phospholipid analysis

Phospholipid concentrations were determined by phosphate assay [13]. To separate the *E. coli* lipid classes for analysis, thin layer chromatography on silica gel G was performed using chloroform/methanol/acetic acid (65:25:8, v/v). To quantitate the phospholipid in each class, phosphate analysis was performed directly on the silica gel scraped from plates charred after spraying with 50% (v/v) sulfuric acid. To determine the molecular species present in the PE fraction, the *t*-butyldimethylsilyl diacylglycerol esters were prepared after eluting the PE fraction from the silica gel (uncharred plates) with chloroform/methanol/water (5:5:1, v/v) and evaporating the solvent. Diacylglycerols were formed by the action of 10 units of phospholipase C from *B. cereus* (Sigma, St. Louis, MO) in 200 μl 0.4 mM zinc chloride, 1 mM β-mercaptoethanol, 200 mM sodium phosphate (pH 7) on 250 nmol PE for 2 h at 37°C with shaking. The solution was extracted 3 times with 1 ml diethyl ether. The combined extracts were washed with sodium sulfate and dried under nitrogen. The *t*-butyldimethylsilyl derivatives were prepared and analyzed by capillary GLC as described by Myher and Kuksis [14] and modified by Pessin and Raben [15].

2.8. Analysis of cross-linked products

Extracted phospholipids [12] from acetic-acid-stopped cross-linking reactions of both whole cells and extracted lipids in multilamellar vesicles were subjected to preparative one-dimensional TLC in chloroform/methanol/acetic acid (65:25:8, v/v) or two-dimensional TLC [3]. Small amounts were subjected to TLC separately to quantitate the extent of the cross-linking reaction by phosphate assay. The dimeric PEs isolated from TLC were subjected to HPLC in 20 mM choline chloride in ethanol/water/hexane (77:13:10, v/v) on four Altex Ultrasphere ODS columns (4.6 × 250 mm) in series. The flow rate was 0.1 ml/min and the chart speed was 1 cm/h. Detection was by absorbance at 205 nm. The HPLC peaks were quantitated by cutting a copy of the chromatogram and weighing the areas under the peaks. All dimeric PEs have similar extinction coefficients, so the peak areas reflect the amount of material under each peak [3]. A few peaks, however, contain more than one dimeric species. The mol% of the dimers, as described by HPLC peak weights, is reported as raw data which includes all deviations from the baseline in the chromatogram, uncorrected for baseline noise or possible non-dimeric reaction products.

In order to quantitate individual dimeric species more specifically, and to identify the dimeric species, the material collected from HPLC was derivitized and subjected to GLC. An internal standard (di 15:0-glycerol) was added to the material in each HPLC peak. Individual peak samples were derivatized to *t*-butyldimethylsilyl diacylglycerols and quantitated by GLC analysis, as described for unreacted PEs under 'Phospholipid analysis'. The amount of each dimer was determined from the amount of the diacylglycerol derivatives of the dimer in the HPLC peaks. When small amounts of the diacylglycerol derivatives of some dimeric species were found in HPLC peaks neighboring the major peak for that species, those amounts were added to the amount in the major peak. The mol% of each dimer is reported as a 'normalized percentage', which was determined by dividing the amount (nmol) of each dimer detected by the total amount (nmol) of the major dimers detected and multiplying by the percent of the total dimers represented by the major dimeric species, assuming a random arrangement. This data treatment was necessitated by the variable background of very small GLC peaks which

made very precise quantitation by GLC of the total crosslinked material impossible. In all cases, the diacylglycerol composition of the pooled cross-linked material was identical to the composition of the unreacted PE molecular species, suggesting that any observed differences in dimer pairings by particular species were not due to differences in the ability of DMS to react with various species.

Peak identification was originally augmented by adding small amounts of radioactive lipids of defined molecular species to the *E. coli* phospholipids before cross-linking and analyzing the pattern of radioactive lipid dimers formed (data not shown). The elution order for the dimers from HPLC could be predicted approximately by assigning each dimer a 'carbon number', which was equal to the number of carbons in the four fatty acyl groups minus 1.5 for each carbon-carbon double bond and approximately 1.3 for each cyclopropane group. Dimers with lower 'carbon numbers' eluted first. In general the peaks were separated by about one half of a 'carbon number'.

2.9. Calculation of nearest neighbor pairs expected for random cross-linking

These calculations were performed essentially as described [8], based on the experimentally determined PE molecular species composition. For homodimers, the predicted mol fraction of dimer is the square of the mol fraction of that molecular species. For heterodimers, the predicted mol fraction is twice the product of the mol fractions of the component molecular species.

3. Results

3.1. Fraction of phospholipid cross-linked and stability of cross-links in whole cells

In all cases in which we have analyzed the molecular species of the dimeric PEs, 2–8% of the total phospholipid (or 3–11% of the total PE) was cross-linked by dimethyl suberimidate. Because Marinetti and Love [16] reported that *E. coli* outer membranes contain an enzyme which breaks down trinitrophenyl PE and because, in whole cells, there are potentially a number of molecules other than PE with which dimethyl suberimidate can react, we first measured the formation of PE dimers both as a function of DMS concentration and of time. This whole cell cross-linking was performed in 100 mM NaCl, 50 mM KH_2PO_4 (pH 8). The amount of dimer formation, as determined by phosphate analysis of the reaction products after separation by TLC, is shown in Fig. 1. These data suggested that dimer breakdown was not occurring to an extent which would preclude dimer analysis after a 10 min cross-linking reaction.

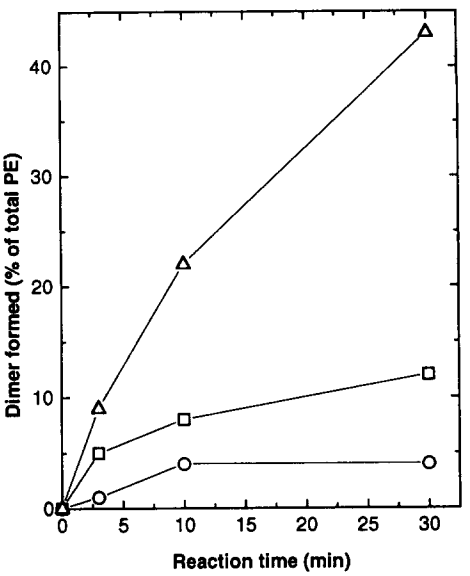

Fig. 1. PE dimer formation in *E. coli* strain AB1623 (whole cells) at pH 8 as a function of the time of reaction with dimethyl suberimidate. Reactions were terminated by addition of glacial acetic acid, followed by lipid extraction and analysis. ○ represents a ratio of DMS to PE of 0.13; □ represents a ratio of 0.86; and △ represents a DMS to PE ratio of 6.47.

3.2. Phosphatidylethanolamine molecular species of strain AB1623

E. coli strain AB1623 is a wild-type strain with regard to phospholipid and fatty acyl synthesis. The phospholipids are in the fluid phase at 37°C. The PE molecular species from strain AB1623 determined by GLC analysis of the *t*-butyldimethylsilyl diacylglycerol esters are shown in Table 1.

3.3. Analysis of dimeric molecular species resulting from cross-linking of PEs of strain AB1623

Whole bacteria or the total phospholipid fraction derived from *E. coli* strain AB1623 and dispersed in

Table 1
Molecular species of PE from *E. coli* strain AB1623

	mol% of total
16:0–16:1	45.0 ± 0.1 [a]
16:1–18:1	20.4 ± 0.8
16:0–18:1 + 18:0–16:1	14.1 ± 0.5
16:1–16:1	5.4 ± 0.9
16:0–17cp	6.0 ± 0.2
18:1–18:1	3.5 ± 0.2
16:0–16:0	3.0 ± 0.3
16:1–17cp	1.5 ± 0.1
18:1–17cp	1.1 ± 0.1

[a] The data represent the mean of quadruplicate samples ± S.D.

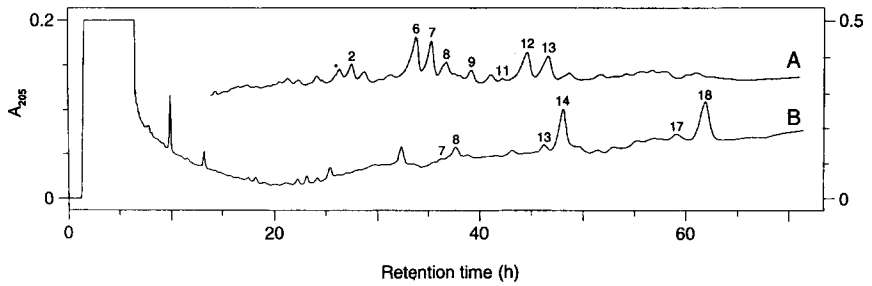

Fig. 2. High-performance liquid chromatogram of PE dimers derived from *E. coli* detected by absorbance at 205 nm. Trace A depicts the species derived from strain AB1623 reacted with dimethyl suberimidate in whole cells at pH 8 and 37°C. Trace B depicts the species derived from strain L8-2 reacted with dimethyl suberimidate in whole cells at pH 8 and 37°C.

multilamellar vesicles were treated with DMS as described in Material and Methods. The dimeric phosphatidylethanolamines from three cross-linking reactions on extracted lipids dispersed in buffer at pH 8 and three cross-linking reactions of whole cells at pH 8 were resolved by HPLC and quantitated. A chromatogram from one of three whole cell samples is shown in Fig. 2A.

Table 2 shows the amounts of each dimeric species or group of dimeric species expected from theoretical calculations based on random pairing of the PE molecular species present in strain AB1623 (Table 1), along with experimental data on the amount of each dimeric species or group of species formed in this natural lipid mixture. The similarity between the amounts of dimers predicted for random mixtures and the experimental

Table 2
Comparison of dimeric species obtained from cross-linking of AB1623 extracted lipids at pH 10 (culture A) with the predicted values based on random cross-linking

Dimeric species	GLC quantitation after HPLC separation				Quantitation of HPLC peak sizes		
	peaks [a]	predicted random mol% [b]	mol% found in ext. lipids [c]	mol% found in whole cells [c]	Peak [d]	mol% found in ext. lipids [e]	mol% found in whole cells [e]
16:0–16:1/16:0–16:1	5, 6, 7	20.2 ± 0.1	19.0 ± 3.0 [f]	20.7 ± 0.6 [f,g]	6	14.6	20.8 ± 2.9
16:0–16:1/16:1–18:1	6, 7, 8	18.4 ± 0.7	22.4 ± 1.1 [i]	20.4 ± 1.6 [f,g]	7	15.1	16.0 ± 2.8
16:0–16:1/16:0–18:1 +16:0–16:1/18:0–16:1	12, 13	12.7 ± 0.5	9.5 ± 2.2 [h]	10.9 ± 1.5 [f,g]	12	10.7	13.1 ± 2.6
16:0–16:1/16:1–16:1	1, 2, 3	4.9 ± 0.1	4.5 ± 0.3 [f]	4.7 ± 0.5 [f,g]	2	5.0	6.0 ± 1.3
16:0–16:1/16:0–17cp	9	5.4 ± 0.2	5.9 ± 1.2 [f]	5.0 ± 1.2 [f,g]	9	4.0	3.6 ± 1.2
16:0–18:1/16:1–18:1 +18:0–16:1/16:1–18:1	12, 13	5.8 ± 0.3	7.4 ± 1.2 [h]	7.6 ± 1.0 [g,h]	13	10.5	10.6 ± 1.5
16:0–16:1/18:1–18:1	13, 14	3.1 ± 0.2	3.9 ± 0.9 [f]	3.8 ± 0.2 [g,h]			
16:1–18:1/16:1–18:1	8, 9	4.2 ± 0.2	3.5 ± 1.2 [f]	3.1 ± 0.6 [g,h]	8	4.8	6.6 ± 4.0
16:0–16:1/16:0–16:0	11	2.7 ± 0.3	1.2 ± 0.1 [i]	1.1 ± 0.1 [g,i]	11	1.3	1.1 ± 0.6

[a] The peak numbers correspond to the peaks in Fig. 2A. Underlined peak numbers represent the peak where the largest amount of this dimer was found.

[b] These predicted values for dimer formation are based on random cross-linking of the molecular species shown in Table 1 (see Materials and methods).

[c] Experimental values representing the percent of each dimer found. The nine dimeric species examined represented 77.4% of the predicted total for dimeric species based onr andom cross-linking and accounted for all species predicted to be present at levels greater than 2%, assuming random cross-linking. The experimental values were normalized so that these nine species accounted for 77.4% of the total, as described in Materials and methods. Three samples were averaged for the extracted phospholipids and three samples were averaged for the whole cells. The values represent the mean ± S.D.

[d] These represent the numbers of the HPLC peaks which were quantitated in terms of peak area by absorbance at 205 nm. See Fig. 2A.

[e] These values are the average areas under the peaks as a percentage of the total area under all peaks. The values represent the mean of duplicate samples for extracted lipids and the mean of 3 samples ± S.D. for whole cells. All deviations from the baseline were included (see Materials and methods).

[f] Not significant at $P = 0.05$ compared to that expected for random cross-linking.

[g] Not significant at $P = 0.05$ compared to that obtained for cross-linking of extracted lipids.

[h] Significant at $P < 0.05$ compared to that expected for random cross-linking.

[i] Significant at $P < 0.01$ compared to that expected for random cross-linking.

Table 3
Major molecylar species of PE from *E. coli* strain L8-2

	mol% of total
18:1–18:1	51.8 ± 2.5 [a]
16:1–18:1	24.4 ± 1.6
16:0–16:1	8.0 ± 0.2
16:0–18:1 + 18:0–16:1	6.8 ± 0.4
18:1–17cp	3.6 ± 0.2
16:0–17cp	3.1 ± 0.2
16:1–16:1	1.4 ± 0.1
16:1–17cp	0.9 ± 0.3

[a] The data represent the mean of quadruplicate samples ± S.D.

data suggests that the PE species in the phospholipids were close to randomly arranged. There were no statistically significant differences between the dimeric species obtained in the extracted phospholipids and in whole cells and only very slightly significant differences between the cross-linking patterns obtained with either of the lipid mixtures and that predicted for random cross-linking. The similarity between whole cells and extracted lipids suggests that there were insignificant differences between the compositions of each of the two monolayers of the inner membrane and the inner monolayer of the outer membrane in the whole cells. The similarity of the experimental values to those predicted for random mixing suggests, of course, that the lipid species in both the extracted phospholipids in multilamellar vesicles at pH 8 and in the whole cells at pH 8 were close to randomly arranged. One species which did seem to be present at significantly lower concentrations than predicted for a random arrangement was the dimer of 16:0–16:1 and 16:0–16:0 (peak 11).

3.4. Phosphatidylethanolamine molecular species of strain L8-2

Strain L8-2 has a β-oxidation defect and a temperature-sensitive mutation involving total fatty acid synthesis. Growth of L8-2 for 2 to 3 generations in *cis*-vaccenate at the restrictive temperature resulted in the PE molecular species shown in Table 3.

3.5. Analysis of dimeric molecular species resulting from cross-linking of PEs of strain L8-2

After cross-linking the L8-2 PEs in both the extracted phospholipids and in whole cells at pH 8, the dimeric species were analyzed by the same method as the AB1623 species. An HPLC elution profile of the

Table 4
Comparison of dimeric species obtained from cross-linking of L8-2 lipids at pH 8 (culture B) with the predicted values based on random cross-linking

Dimeric species	GLC quantitation after HPLC separation				Quantitation of HPLC peak sizes		
	peaks [a]	predicted random mol% [b]	mol% found in ext. lipids [c]	mol% found in whole cell [c]	peak [d]	mol% found in ext. lipids [e]	mol% found in whole cells [e]
16:1–18:1/18:1–18:1	13, 14, 15	25.3 ± 2.1	21.6 ± 7.5 [f]	23.2 ± 9.8 [f,g]	14	14.6 ± 0.9	21.6 ± 1.8
18:1–18:1/18:1–18:1	18	26.8 ± 1.8	28.1 ± 4.9 [f]	25.8 ± 7.6 [f,g]	18	25.5 ± 3.0	29.7 ± 4.2
16:1–18:1/16:1–18:1	8, 9	5.9 ± 0.5	9.1 ± 3.0 [f]	12.3 ± 6.4 [f,g]	8	5.4 ± 1.8	7.0 ± 1.5
18:1–18:1/16:0–16:1	13	8.3 ± 0.5	3.7 ± 0.9 [h]	2.8 ± 2.6 [f,g]			
16:0–18:1/16:1–18:1 + 18:0–16:1/16:1–18:1	12, 13, 14	3.3 ± 0.3	5.6 ± 2.5 [f]	7.8 ± 5.3 [f,g]	13	3.7 ± 0.7	4.1 ± 1.5
16:0–18:1/18:1–18:1 + 18:0–16:1/18:1–18:1	16, 17	7.0 ± 0.5	8.8 ± 2.9 [f]	6.5 ± 4.1 [f,g]	17	5.2 ± 2.0	5.0 ± 1.2
16:0–16:1/16:1–18:1	6, 7	5.8 ± 0.4	5.4 ± 1.2 [f]	3.9 ± 4.4 [f,g]	7	1.8 ± 1.6	2.0 ± 1.4

[a] The peak numbers correspond to the peaks in Fig. 2B. Underlined peak numbers represent the peak where the largest amount of this dimer was found.
[b] These predicted values for dimer formation are based on random cross-linking of the molecular species shown in Table 3 (see Materials and methods).
[c] Experimental values representing the percent of each dimer found. The seven species examined represented 82.4% of the predicted total for dimeric species based on random cross-linking and accounted for all species predicted to be present at levels greater than 4%, assuming random cross-linking. The experimental values were normalized so that these seven species accounted for 82.4% of the total, as described in Materials and methods. Five samples were averaged. The values represent the mean ± S.D.
[d] These represent the numbers of the HPLC peaks which were quantitated in terms of peak size. See Fig. 2B.
[e] These values are the average areas under the peaks as a percentage of the total area under all peaks. In each case, the values represent the mean of 4 samples ± S.D. All deviations from the baseline were included (see Materials and methods).
[f] Not significant at $P = 0.05$ compared to that expected for random cross-linking.
[g] Not significant at $P = 0.05$ compared to that obtained for cross-linking of extracted lipids.
[h] Significant at $P < 0.01$ compared to that expected for random cross-linking.

Table 5
Ratio of experimentally obtained amounts of major dimers of L8-2 to the amounts predicted based on random mixing

	Ratio of experimental to random predicted value	
GLC data	18:1–18:1/18:1–18:1	16:1–18:1/18:1–18:1
Extracted lipids, pH 8	1.05 ± 0.20	0.85 ± 0.30
Whole cells, pH 8	0.96 ± 0.29	0.88 ± 0.37
HPLC peak area data	peak 18 (18:1–18:1/18:1–18:1)	peak 14 (16:1–18:1/18:1–18:1)
Extracted lipids, pH 8	0.95 ± 0.13	0.58 ± 0.06
Whole cells, pH 8	1.11 ± 0.17	0.85 ± 0.10

dimeric species formed in whole cells is shown in Fig. 2B. The amounts of the major dimeric species formed as compared with predicted values, based on random cross-linking, are shown in Table 4. Again, the results for the whole cells and extracted lipids were similar, suggesting a lack of compositional asymmetry among membranes and monolayers. Also, again the deviations from random for the whole cells and the extracted phospholipid mixtures based on GLC analysis were not statistically significant. However, the ratio of di 18:1 homodimer to the predicted value based on random cross-linking was consistently greater than the ratio of the amount of the second most common pair, 16:1–18:1/18:1–18:1 to its predicted value based on random cross-linking. This can be seen by examination of the calculated data shown in Table 5. Both data from GLC analysis and measurements of HPLC peak areas are shown. For peaks 14 and 18, which represent the 16:1–18:1/18:1–18:1 and the 18:1–18:1/18:1–18:1 dimers, respectively, the HPLC peak sizes should have been an accurate indication of dimeric amount since neither of these peaks contains significant amounts of other dimers, and very little of either of these species were found in neighboring peaks.

4. Discussion

Dimethylsuberimidate cross-linking has been used to determine the arrangement of phosphatidylethanolamine molecular species in *E. coli* membranes. The cross-linking patterns obtained from lipids in whole cells and in extracted lipids reconstituted into multilamellar vesicles were similar. The *E. coli* outer membrane is highly asymmetric with the outer monolayer lipid consisting of lipopolysaccharide and the inner monolayer composed of phospholipids, while both monolayers of the inner membrane are composed of phospholipids [17]. Our findings of similar cross-linking patterns for whole cells and extracted lipids reconstituted into vesicles suggest that there is little difference in the PE molecular species composition of the three lipid monolayers (the inner and outer monolayers of the inner membrane and the inner monolayer of the outer membrane) which contain significant amounts of PE.

No large deviations from random mixing of the PE molecular species were found in wild-type strain AB1623, either in whole cells or in extracted lipids which were reconstituted into multilamellar vesicles. In whole cells or in lipids extracted from whole cells of *E. coli* strain L8-2 with membranes enriched in divaccenoyl PE, the PE molecular species were also very close to randomly arranged. However, a consistent, slight tendency of divaccenoyl species to pair with like species as compared to pairing with the second most common species, 18:1–16:1 PE, was observed in both extracted lipids and in whole cells. These results tend to corroborate the results of Baldassare et al. [5], who found that TEMPO partition measurements on extracted lipids derived from strain L8-2 grown with vaccenate showed a break at 46°C. These authors postulated that this corresponded to the breakup of quasi-crystalline arrays of divaccenoyl lipids in the liquid crystalline phase.

A major problem in the analyses is obtaining very clean, statistically significant data. We have reported our values for quantitation of the dimers both as HPLC peak areas and as amounts determined by derivitizing the material in each peak and quantitating by GLC analysis. Analysis of diacylglycerol derivatives derived from the dimers should have allowed precise quantitation of the dimeric species. However, there were a number of manipulations which had to be done in the analyses. During this handling, small amounts of contaminants were undoubtedly introduced, which made very precise quantitation of the total dimeric pool by GLC difficult. Thus we have 'normalized' our GLC data to the pool of 70–85% of the dimers which we can positively identify and quantitate with confidence. In doing this, we have implicitly made the assumption that these major dimeric species, as a group, show no overall preference between cross-linking to other major dimers or cross-linking to minor dimers. In contrast to the GLC data, the HPLC peak area data were reported without any corrections. This simpler analysis is subject to the potential problem of minor constituents co-migrating with the major dimeric species. However, this potential problem should have been minimal since we have demonstrated that the extinction coefficients of the dimeric species are high compared to underivatized species [3]. In some cases, however, if there was significant baseline noise in the HPLC absorbance detection, the reported sizes of the peaks (taken as a

percent of the total peak areas) may be smaller than their true values.

We have previously observed, using the same cross-linking technique in model membranes, that, in fluid-phase mixtures of di 16:0 PE and di 18:1 (dioleoyl) PE, there was a small amount of immiscibility [8]. Similarly, in a mixture of fluid-phase di 14:0 and di 18:0 phospholipids which were linked by disulfide bonds, a slight preference for clustering of species with the same acyl chains was observed by Krisovitch and Regen [18]. These model system data contrast with the three other binary fluid-phase mixtures (dilauroyl PE/dielaidoyl PE, dimyristoyl PE/dielaidoyl PE, and stearoyl, decanoyl PE/dimyristoyl PE) which we have examined, in which complete miscibility was observed [8,9]. It is interesting to note that, thus far, our technique has detected fluid-phase immiscibility only in mixtures containing di-*cis*-18:1 PEs. Importantly and as expected, however, we observed substantial immiscibility in a gel phase mixture which exhibited non-ideal mixing as evidenced by its phase diagram [9], demonstrating that the DMS cross-linking method can detect immiscibility where it exists.

Also in previous work, we used steady-state fluorescence polarization to characterize the gel-fluid phase partition characteristics of parinaroyl PEs and PGs. Fluorescence polarization data on two parinaroyl PEs and two parinaroyl PGs in mixtures of solid-phase DMPE and fluid-phase dilinoleoyl PG demonstrated that the partitioning of these probes was not strongly dependent on head group [19]; instead, acyl chain identity rather than head group identity was the primary factor affecting the organization of gel-fluid PE/PG mixtures, at least in the absence of proteins. Thus, if results on gel-fluid mixtures can be extended to fluid mixtures, substantial clustering of molecular species based on head group similarity, as has been seen by Rodgers and Glaser [1] in erythrocytes and by De Bony et al. [2] in *M. luteus*, was unlikely to be found in *E. coli*. Taken together with the present data, our results suggest subtle, if any, domain formation in the isolated, reconstituted *E. coli* lipids which we have examined. In whole cells where membrane proteins are present, no further clustering of particular molecular species within the PE class was detected in the *E. coli* strains examined.

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6. References

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