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# INTERACTIONS BETWEEN LIPOPOLYSACCHARIDE AND PHOSPHATIDYLETHANOLAMINE IN MOLECULAR MONOLAYERS

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## Summary

Lipopolysaccharide and phosphatidylethanolamine are the two major lipid constituents of the membrane of *Salmonella typhimurium*. Interactions between the purified lipopolysaccharide and phosphatidylethanolamine were studied in molecular monolayers at air-water interfaces.

The equilibrium surface pressures of mixed films of lipopolysaccharide and phosphatidylethanolamine were determined as a function of the film composition. The plot of the equilibrium surface pressure vs. the area occupied by phosphatidylethanolamine molecules exhibited two distinct regions. Below a phosphatidylethanolamine surface concentration at which 55% of the surface was occupied by phosphatidylethanolamine molecules, the equilibrium pressure was invariant and had the value of a pure lipopolysaccharide monolayer at maximum compression. At phosphatidylethanolamine surface concentrations in excess of 55% surface area occupation (phosphatidylethanolamine/lipopolysaccharide (mol/mol) > 16), the equilibrium surface pressure was a function of the surface concentration of phosphatidylethanolamine. The results suggest a simple model in which lipopolysaccharide and phosphatidylethanolamine form a complex in which each lipopolysaccharide molecule is surrounded ('lipidated') by a shell of approx. 16 phosphatidylethanolamine molecules.

## Introduction

Biological membranes contain a large number of amphipathic molecules and it is likely that specific interactions between these molecules play important roles in membrane structure and function. These interactions have been defined

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best in the case of protein-lipid interactions, which have been implicated in the function of several membrane enzyme systems [1]. In the case of cytochrome oxidase, it has been shown directly that a fraction of the phospholipid is closely associated with the protein, presumably as a shell of 'boundary lipid' [2] and it is likely that similar lipid-protein associations obtain for other intrinsic membrane proteins. In contrast, it is not known whether specific associations occur between lipid molecules within the basic phospholipid matrix of the membrane. The present study describes interactions between bacterial membrane glycolipid and phospholipid components.

The cell envelope of *Salmonella typhimurium* and other Gram-negative bacteria contains a complex glycolipid (lipopolysaccharide) consisting of covalently cross-linked monomeric units whose structure is shown in Fig. 1. Each monomer contains a non-polar region ('Lipid A') covalently linked to a complex polysaccharide.

The cell envelope of these organisms contains two membranes. Most of the lipopolysaccharide is located in the outer membrane where it occurs together with phospholipids and proteins. A small amount of lipopolysaccharide is also present in the inner (cytoplasmic) membrane where it is part of a membrane-bound system, composed of lipopolysaccharide, phospholipid and glycosyltransferase enzymes, that is responsible for biosynthesis of the polysaccharide portion of the lipopolysaccharide molecule (see ref. 3, for review). In vitro reconstitution studies using purified transferase enzymes, lipopolysaccharide (LPS) [1] and phosphatidylethanolamine (PE) have indicated that the initial stage in reassociation of these components is formation of a mixed lipopolysaccharide-phosphatidylethanolamine structure, as shown in the following

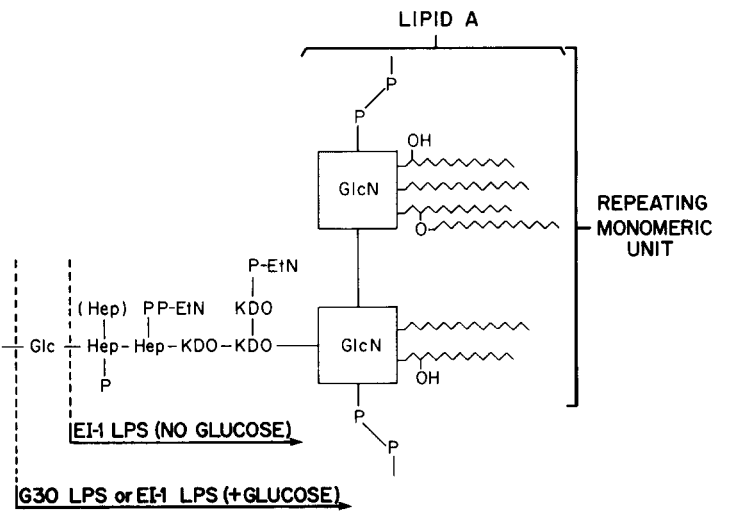



Fig. 1. Structure of lipopolysaccharide from *S. typhimurium* strain G30. The lipopolysaccharide molecule is composed of three of the monomeric units shown in the figure, presumably held together by phosphodiester bonds [12]. Abbreviations: EtN, ethanolamine; Hep, L-glycero-D-mannoheptosyl; Glc, glucosyl; GlcN, glucosaminyl; P, phosphate; KDO, 2-keto-3-deoxyoctonyl. The fatty acids are indicated by the zig-zag lines.

scheme:

$$\text{LPS} + \text{PE} \rightarrow \text{LPS-PE} \quad \text{(I)}$$

$$\text{Enzyme} + \text{LPS-PE} \rightarrow \text{Enzyme-LPS-PE} \quad \text{(II)}$$

The above sequence has been demonstrated in aqueous solution by isolation of the indicated intermediate complexes [4]. Reconstitution also has been shown using monolayer techniques [5,6] with functional reassembly being indicated by restoration of transferase enzyme activity in oriented films at air-water interfaces. The restoration of function suggests that the molecular organization of the components in the reconstituted system is probably similar to their organization within the membrane.

In this communication, we report preliminary studies of the nature of the lipopolysaccharide-phosphatidylethanolamine complex. These studies, using the monolayer system, suggest a model in which each lipopolysaccharide molecule is surrounded ('lipidated') by approx. 16 phosphatidylethanolamine molecules in the plane of the monolayer.

## Materials and Methods

*(a) Preparation of lipopolysaccharide*

Galactose-deficient lipopolysaccharide was isolated and purified from *S. typhimurium* G30 (deficient in UDP galactose-4-epimerase) as previously described [5]. Concentrations of lipopolysaccharide were determined from heptose analysis. One molecule of lipopolysaccharide was taken as the trimeric form containing a total of six heptose residues [5]. Tritium-labeled G30 lipopolysaccharide was prepared by growing strain G30 under conditions identical to the unlabeled material except that the medium contained *N*-acetyl-D-[1-$^3$H]-glucosamine (Amersham/Searle). Label was present for two generations of growth. The lipopolysaccharide from the labeled bacteria was purified by procedures identical to those used to purify the unlabeled material. The labeled lipopolysaccharide mixtures used in the experiments were prepared by mixing the appropriate amounts of labeled and unlabeled material. Specific activities (determined by heptose assays) ranged from 24 000 to 140 000 dpm/nmol lipopolysaccharide.

*(b) Preparation of phosphatidylethanolamine*

Phosphatidylethanolamine from *S. typhimurium* G30A was prepared as previously described [4] with the modification that a silicic acid column chromatography purification step [7] followed the DEAE-cellulose chromatography step of the original purification scheme. The purified material was subjected to thin-layer chromatography in solvents I and II (see below). When the plates were stained with iodine vapor, both solvent developing systems showed a single spot comigrating with authentic phosphatidylethanolamine. Concentration of phosphatidylethanolamine was determined by phosphate analysis.

Labeled phosphatidylethanolamine was prepared from cells of strain G30A grown in the presence of [2-$^{14}$C]acetate (New England Nuclear) for two genera-

tions. Except for the presence of label, the growth conditions and purification procedure were identical to those used to prepare the unlabeled phosphatidylethanolamine. When the purified labeled material was analyzed by thin-layer chromatography as described above the [$^{14}$C]phosphatidylethanolamine showed a single peak of radioactivity comigrating (>95%) with authentic phosphatidylethanolamine. The [$^{14}$C]phosphatidylethanolamine was mixed with unlabeled phosphatidylethanolamine to prepare mixtures of appropriate specific activity. Specific activities ranged from 2000 to 20 000 dpm/nmol.

*(c) Analytical procedures*

Heptose was measured as described by Kent and Osborn [8]. Total phosphate was determined by the method of Ames and Dubin [9]. Thin-layer chromatography of phospholipids was performed on silica gel plates (Eastman) in the following solvent systems: solvent I, $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 1, v/v); solvent II, $CHCl_3/CH_3OH$/glacial acetic acid (7 : 3 : 1, v/v). Paper chromatography of $^3$H-labeled lipopolysaccharide was performed on Whatman No. 1 filter paper developed in isobutyric acid/1 M $NH_4OH$ (5 : 3, v/v) as described by Boman and Monner [10]. Authentic G30 lipopolysaccharide was co-chromatographed with the labeled sample. After developing, the chromatogram was cut, one half was stained with silver nitrate-periodate [11] to visualize the standard and the other half cut into strips and counted to determine the distribution of label.

*(d) Monolayer experiments*

*(1) Equipment.* The monolayer experiments were performed with the three compartment trough and apparatus described previously [5]. The only modification of the apparatus was the replacement of all glass components by Teflon. The movable frame in which the monolayer was formed was machined out of a single block of teflon and had a fixed area $1.91 \cdot 10^{15}$ nm$^2$. Preparation of other materials and reagents and details of the technique are given by Romeo et al. [5]. The subsolution in all experiments was 20 mM Tris-HCl, pH 8.5, and the temperature was maintained at 27.5 ± 0.5°C.

*(2) Penetration experiments.* Known amounts of phosphatidylethanolamine dissolved in $CHCl_3/CH_3OH$ (85 : 15, v/v) were applied to the surface enclosed by the teflon frame using a calibrated Hamilton microsyringe. Surface pressure was monitored continuously as described previously [5]. After addition of the phosphatidylethanolamine, a solution of lipopolysaccharide was injected into the subsolution to a final concentration of 5 $\mu$M and the subsolution was stirred with a magnetic stirrer. Penetration was followed by monitoring the surface pressure. Equilibrium pressure was considered established when the surface pressure remained constant for at least 4 h. To test whether such films were at maximal compression, a teflon barrier at one edge of the fixed area frame was moved slowly over the surface so that the surface area was decreased. It was found without exception that films which had reached a constant surface pressure were also at maximal compression since decreasing the area of such films did not increase the pressure.

*(3) Quantitation of phosphatidylethanolamine and lipopolysaccharide in mixed films.* The composition of the mixed films of phosphatidylethanol-

amine and lipopolysaccharide was determined using a double label technique. Films were formed and penetration of lipopolysaccharide performed as described above using [$^{14}$C]phosphatidylethanolamine and $^{3}$H-labeled lipopolysaccharide of known specific activities.

To remove the unabsorbed $^{3}$H-labeled lipopolysaccharide from the subsolution the following washing regimen was followed. After penetration to constant pressure the film was transferred to the middle compartment of the three compartment trough, the subsolution stirred to remove material not firmly bound within the monolayer, and the film then moved to the surface of the third compartment. The subsolution from the first two compartments was removed by aspiration and fresh subsolution was added. The film was then moved back to the first compartment after stirring the subsolution in the middle compartment. This regimen reduced the background contribution of $^{3}$H-labeled lipopolysaccharide of the final subsolution to levels from 8- to 60-fold lower than the amount recovered in the monolayer films. This range reflects the difference in film compositions rather than the effectiveness of washing. Surface pressure was monitored continuously during the manipulations of the monolayer film to confirm that the integrity of the film was maintained and that washing did not lead to desorption of material as would be indicated by a drop in surface pressure.

For direct analysis of the film the surface layer was removed by aspiration through a capillary tube. Quantitative recovery (see below) was achieved by sweeping the surface layer to a corner of the frame with a Teflon bar during the aspiration procedure.

Immediately following the recovery of the film, aliquots of the subsolution were removed that were equal to the volume removed with the film during the aspiration (0.5—0.8 ml) and the analysis of the film was corrected for material present in the subsolution. The samples were transferred quantitatively to liquid scintillation vials by successive washings with a toluene-based scintillation fluid containing 15% Biosolv (Beckman). The aqueous content of the samples was adjusted to 5% by addition of subsolution buffer so that analysis by double label counting was performed at constant quenching. Samples were counted on a Beckman LS230 scintillation counter and spillover corrections were made using standards prepared in identical scintillation cocktails with identical quenching.

In control experiments, the efficiency of recovery was determined under several different conditions. In the first set of experiments, known amounts of [$^{14}$C]phosphatidylethanolamine, covering the entire range of phosphatidylethanolamine concentrations used in the penetration experiments, were applied to the surface of a clean subphase. When the surface film then was removed by aspiration and counted there was consistent recovery of 90—95% of the label in the aspirated sample and less than 0.1% was found in the subphase. Extensive washing with chloroform of the Teflon components forming the film containment frame recovered an additional 3—5% of the total counts. In a second series of control experiments, unlabeled lipopolysaccharide was injected into the subphase after the labeled phosphatidylethanolamine was placed on the surface, and films were recovered after the equilibrium pressure had been reached. With high phosphatidylethanolamine films ($\alpha_{PE} > 0.3$, Eqn. 1, see

below), 90% of the label was recovered in the film, 1% or less had desorbed and was recovered in the subphase and about 5% was associated with the apparatus. At very low phosphatidylethanolamine concentrations ($\alpha_{PE} < 0.3$), 75% of the radioactivity was recovered in the film and 15% had desorbed into the subphase. Values for $N$ in calculations of $\alpha_{PE}$ (Eqn. 1) were corrected for these losses. In another set of preliminary experiments only a fraction of a mixed [$^{14}$C]phosphatidylethanolamine-$^{3}$H-labeled lipopolysaccharide film was removed by aspirating for a short time so that surface pressure did not decrease to zero or by permitting the equilibrium film to partially escape beyond the boundaries of the movable frame. The $^{14}C/^{3}H$ ratio in the aspirate was the same (±15%) as in films in which the film was completely removed.

The limiting area per molecule of phosphatidylethanolamine was determined from monolayer isotherms at 27.5°C as described previously [5]. The area per molecule of G30A phosphatidylethanolamine at collapse ($A_{PE}$) was 0.54 nm$^2$ with a collapse pressure of 44 dynes · cm$^{-1}$.

## Results

*Formation of lipopolysaccharide-phosphatidylethanolamine monolayers*

In order to study the interaction between lipopolysaccharide and phosphatidylethanolamine, a film of phosphatidylethanolamine was formed on the surface of an aqueous subphase and lipopolysaccharide was injected into the subsolution. Surface pressure was recorded continuously as lipopolysaccharide penetrated into the surface layer. The rate of change of surface pressure was dependent upon the surface concentration of phosphatidylethanolamine in the film, the concentration of lipopolysaccharide in the subphase and the temperature of the system, as previously reported [5]. After a period of time, a surface pressure (equilibrium pressure, $\pi_E$) was reached which was invariant with time and did not change when the film was moved to a subphase not containing lipopolysaccharide.

*Identification of the penetrating species as lipopolysaccharide*

Since less than 1% of the total lipopolysaccharide in the subphase enters the film it was necessary to demonstrate that the labeled material in the film was indeed lipopolysaccharide and not some minor labeled contaminant. Chromatography of labeled material from an equilibrium film showed a single radioactive peak that co-migrated with chemically characterized G30 lipopolysaccharide.

*Fractional composition of two-component films*

There are several ways of expressing the results of experiments in which the final surface pressure of two-component films is measured as a function of composition. The most frequent method is to use the initial pressure of the single component film as an index of surface concentration. Such representations, however, tend to give extra weight to conditions in which the initial component occupies a major fraction of the available surface area, due to the intrinsic shape of the isotherm. This distortion is avoided by expressing the surface composition in terms of the fraction of the surface area occupied by

each of the components in the monolayer at equilibrium.

The fractional surface area of phosphatidylethanolamine, $\alpha_{PE}$, is defined as:

$$\alpha_{PE} = N_{PE} \cdot \frac{A_{PE}}{A_T} \quad (1)$$

where $N_{PE}$ is the number of molecules of phosphatidylethanolamine in the film, $A_{PE}$ is the surface area per molecule of phosphatidylethanolamine at maximal compression (0.54 $nm^2$) and $A_T$ is the total area of the film ($1.91 \cdot 10^{15}$ $nm^2$). $\alpha_{PE}$ was calculated from the amount of phosphatidylethanolamine on the surface (see Materials and Methods).

The fractional surface area of lipopolysaccharide, $\alpha_{LPS}$, is related to $\alpha_{PE}$ by the expression

$$\alpha_{LPS} = 1 - \alpha_{PE} \quad (2)$$

This formulation presumes that the equilibrium films are in a state of maximal compression, and that the area occupied by each molecule of phosphatidylethanolamine in maximally compressed films containing both lipopolysaccharide and phosphatidylethanolamine is the same as in a maximally compressed film of pure phosphatidylethanolamine. To validate these assumptions, two criteria must be met. First, the final films must be at maximal compression, so that the total surface area is occupied by phosphatidylethanolamine and lipopolysaccharide. This was demonstrated directly by showing that there was no further increase in pressure when the area of the films was mechanically decreased by means of a movable surface barrier. Second, the area occupied by each lipopolysaccharide molecule and each phosphatidylethanolamine molecule in the mixed equilibrium film must be independent of the composition of the film (i.e. interaction between phosphatidylethanolamine and lipopolysaccharide does not significantly alter their molecular dimensions within the plane of the film). Support for this assumption is given in the following section.

*Relation between film composition and* $\alpha_{PE}$

If the area occupied by each lipopolysaccharide molecule and each phosphatidylethanolamine molecule in the mixed film is independent of the composition of the film, then the following relationship will hold for all film compositions:

$$A_T = N_{PE} \cdot A_{PE} + N_{LPS} \cdot A_{LPS} \quad (3)$$

where $A_{LPS}$ and $A_{PE}$ are the areas per molecule of lipopolysaccharide and phosphatidylethanolamine, respectively, and $N_{PE}$ and $N_{LPS}$ are the number of molecules of each species in the equilibrium film. Eqn. 3 predicts that a direct relationship exists between $N_{LPS}$ and $\alpha_{PE}$ (i.e. that $\alpha_{PE} - 1 = K \cdot N_{LPS}$, where $K = -A_{LPS} \cdot A_T^{-1}$).

This prediction was confirmed by directly measuring the amounts of lipopolysaccharide and phosphatidylethanolamine in equilibrium films of varying compositions and plotting the amount of lipopolysaccharide as a function of $\alpha_{PE}$ (Fig. 2). The plot shows that the amount of lipopolysaccharide in the film was directly proportional to $\alpha_{PE}$ across the entire range of concentrations. This experimental confirmation of Eqn. 3 validates the use of $\alpha_{PE}$ (and $\alpha_{LPS}$) to

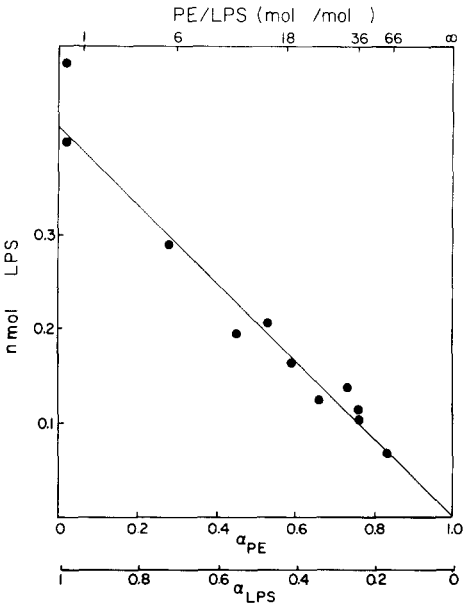



Fig. 2. Quantitation of G30 lipopolysaccharide and phosphatidylethanolamine in equilibrium films. Various amounts of [$^{14}$C]phosphatidylethanolamine were applied to the surface of the monolayer apparatus. A solution of $^{3}$H-labeled G30 lipopolysaccharide was injected into the subphase. At equilibrium ($\pi_E$) the film was recovered after moving it to a clean subphase. The recovered film was counted and the amount of phosphatidylethanolamine and lipopolysaccharide in the film was determined from the known specific activities as described in the text. The amount of lipopolysaccharide in the film is shown as a function of the phosphatidylethanolamine present in the film. $\alpha_{PE}$ and $\alpha_{LPS}$ were calculated as described in the text. At the top of the figure is indicated the molar ratio of phosphatidylethanolamine to lipopolysaccharide in the films.

generate the pressure-composition diagram. (See Appendix for more extensive treatment of Eqn. 3).

From Fig. 2 and Eqn. 3, the molecular area of G30 LPS ($A_{LPS}$) in the plane of the film was calculated to be 7.60 ± 0.80 nm$^2$.

*Relation of equilibrium pressure to film composition*

To study the relation of $\pi_E$ to surface composition, phosphatidylethanolamine films were formed at different initial surface pressures, ranging from 0 dyne · cm$^{-1}$ (0 nmol phosphatidylethanolamine) to 44 dynes · cm$^{-1}$ (5.88 nmol phosphatidylethanolamine). Lipopolysaccharide was allowed to penetrate as described above and $\pi_E$ was measured after completion of penetration. As shown in Fig. 3 $\pi_E$ remained nearly constant as the fraction of phosphatidylethanolamine in the monolayer was increased from $\alpha_{PE} = 0$ to $\alpha_{PE} = 0.55$ (LPS/PE (mol/mol) = 1/16). At this point ($\alpha_{PE} = 0.55$) there was an abrupt increase in slope of the plot of $\pi_E$ vs. $\alpha_{PE}$. Above $\alpha_{PE}$ of 0.55 there was a proportional increase of both parameters with $\pi_E$ approaching the surface pressure of a pure film of phosphatidylethanolamine (44 dynes · cm$^{-1}$) as $\alpha_{PE}$ approached 1.0.

As discussed in the following section, these results suggest that a complex of phosphatidylethanolamine and lipopolysaccharide with a stoichiometry of approx. 16 molecules of phosphatidylethanolamine per molecule of lipopolysaccharide is formed within the mixed films, with the regions below and above $\alpha_{PE} = 0.55$ representing regions of excess lipopolysaccharide and excess phosphatidylethanolamine, respectively.

## Discussion

*(i) Interpretation of the pressure vs. composition diagram*

The pressure vs. composition diagram shown in Fig. 3 can be divided into lipopolysaccharide-rich and phosphatidylethanolamine-rich regions which differ markedly in their characteristics.

In the region between $\alpha_{PE} = 0$ and $\alpha_{PE} = 0.55$, where the film is lipopolysaccharide rich, the curve is relatively insensitive to composition of the film. Several possibilities exist for the organization of the film in this region. (a) Lipopolysaccharide and phosphatidylethanolamine may exist in separate non-interacting phases. This probably can be ruled out by the observed incompressibility of the films since a separate phosphatidylethanolamine phase could be further compressed at these low surface pressures [5]. This would lead to an increased surface pressure on compression of the two-phase film, a result that was not observed. (b) Phosphatidylethanolamine molecules may exist in a complex with lipopolysaccharide molecules. The resulting LPS-PE complexes in turn may reside in a separate phase or may interact with uncomplexed lipopolysaccharide molecules with a similar excess surface free energy to lipopolysaccharide alone. Either situation is consistent with the observation that the $\pi_E$ of such mixtures was similar to that of lipopolysaccharide alone. The idea that lipopolysaccharide and phosphatidylethanolamine are interacting in this lipopolysaccharide-rich domain is supported by preliminary NMR studies (data not shown) which demonstrated that the spectra of mixtures of phosphatidyl-

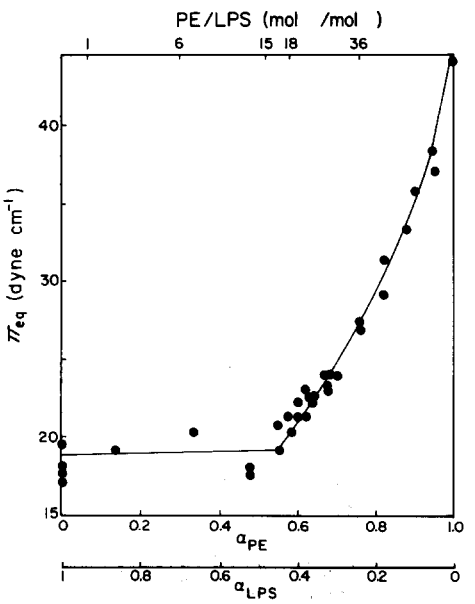



Fig. 3. Monolayer diagram of mixed films of G30 lipopolysaccharide and G30A phosphatidylethanolamine. The equilibrium pressure, $\pi_E$, is shown as a function of the composition of the film expressed as the fraction of the area of the film occupied by phosphatidylethanolamine ($\alpha_{PE}$) and the fraction of the area available to lipopolysaccharide molecules ($\alpha_{LPS}$). The calculations of $\alpha_{PE}$ and $\alpha_{LPS}$ are described in the text.

ethanolamine and lipopolysaccharide in this composition range (PE/LPS < 16) are different than the sum of the spectra of the two individual components.

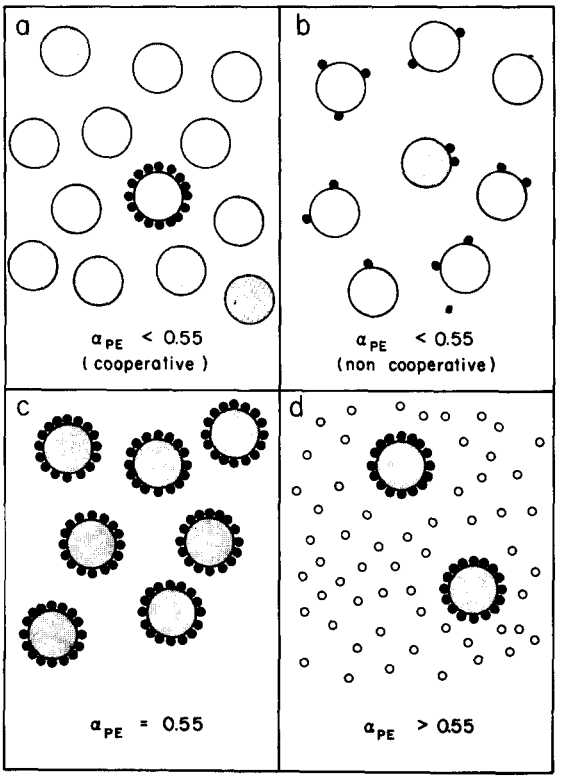
In the region from $\alpha_{PE} = 0.55$ to $\alpha_{PE} = 1.0$ ($N_{PE}/N_{LPS}$ between 16 : 1 and $\infty$) where the film is phosphatidylethanolamine rich, equilibrium pressure was a function of the composition of the film, suggesting simple mixing of the components (phosphatidylethanolamine and lipopolysaccharide-phosphatidylethanolamine complex). In this region, the lipopolysaccharide-phosphatidylethanolamine complex can be viewed as a solute in a matrix of phosphatidylethanolamine molecules. The progressive increase in $\pi_E$, approaching $\pi_E$ for a pure phosphatidylethanolamine film (44 dynes · $cm^{-1}$) as phosphatidylethanolamine/lipopolysaccharide approaches infinity, is ascribed to the increased number of uncomplexed phosphatidylethanolamine molecules in the film. Whether the mixing is ideal (i.e. interactions between phosphatidylethanolamine and LPS-PE complexes are completely equivalent to interactions between phosphatidylethanolamine and phosphatidylethanolamine) cannot be determined with certainty.

*(ii) A model of the phosphatidylethanolamine-lipopolysaccharide interaction*

The data shown in Fig. 3 are consistent with the following model (illustrated in Fig. 4). As phosphatidylethanolamine is added to a matrix of pure lipopolysaccharide, a complex between phosphatidylethanolamine and lipopolysaccharide is formed. Below a phosphatidylethanolamine/lipopolysaccharide molar ratio of 16/1 all phosphatidylethanolamine molecules are complexed with lipopolysaccharide so that lipopolysaccharide-phosphatidylethanolamine complexes and uncomplexed lipopolysaccharide are both present within the monolayer. At the ratio of 16 : 1 the lipopolysaccharide-phosphatidylethanolamine monolayer consists of the homogeneous complex. As more phosphatidylethanolamine is added, the surface concentration of the complexes if progressively diluted until the mixed monolayer eventually approaches a pure phosphatidylethanolamine system.

In the simplest model the complex can be thought of as a molecule of lipopolysaccharide surrounded by a shell of phosphatidylethanolamine molecules (Fig. 4c). The area of a lipopolysaccharide molecule within the film is 7.60 $nm^2$ (from Fig. 2) and the area of a phosphatidylethanolamine molecule is 0.54 $nm^2$ [5]. If we assume that these areas can be treated as discs within the plane of the membrane and, as argued earlier, that the area of the complex is equal to the sum of its components, the minimum number of phosphatidylethanolamine molecules required to 'lipidate' a lipopolysaccharide molecule is 15. The monolayer studies suggest that the stoichiometry of the putative complex is 16 : 1, in good agreement with this simple model. Fig. 4 shows the hypothetical organization of the complex in the three regions of the composition diagram. The data do not indicate whether the binding of phosphatidylethanolamine to lipopolysaccharide is cooperative (Fig. 4a) or non-cooperative (Fig. 5b).

It should be noted that the molecular surface area of lipopolysaccharide from *S. typhimurium* strain EI-1 determined previously in this laboratory [5] was about 2.5 $nm^2$, significantly smaller than that of lipopolysaccharide from strain G30 reported here. We have since repeated and confirmed the results of



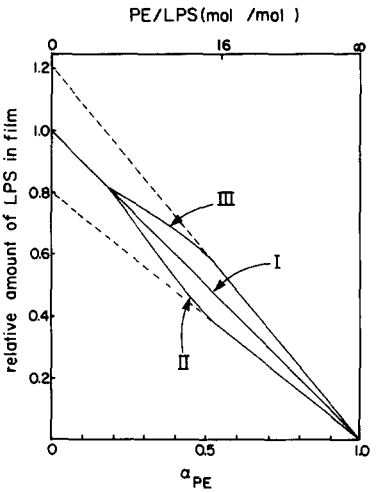




Fig. 4. Models of suggested organization of monolayers in different regions of the $\pi_E/\alpha$ diagram. See text for definition of $\alpha_{PE}$. (a) $0 < \alpha_{PE} < 0.55$, interaction between phosphatidylethanolamine and lipopolysaccharide is cooperative; (b) $0 < \alpha_{PE} < 0.55$, interaction between phosphatidylethanolamine and lipopolysaccharide is non-cooperative; (c) $\alpha_{PE} = 0.55$; (d) $0.55 < \alpha_{PE} < 1$.

Fig. 5. Amount of lipopolysacchride in fixed area films as a function of $\alpha_{PE}$. Relative amount of lipopolysaccharide in film is defined as $T_{N_{LPS}}$ (from Eqn. 7)/$T_{N_{LPS}}$ (from Eqn. 3). Curve I, $\delta = 0$; Curve II, $\delta = +0.2 \cdot (A_{LPS} + 16 \cdot A_{PE})$; Curve III, $\delta = -0.2 \cdot (A_{LPS} + 16 \cdot A_{PE})$.

the EI-1 lipopolysaccharide experiments. This discrepancy may be explained by the fact that the lipopolysaccharide of strain EI-1 terminates in heptose when grown in the absence of glucose whereas the G30 lipopolysaccharide used in the present study terminates in glucose (see Fig. 1). The radioactive EI-1 lipopolysaccharide used for quantitation of surface concentration in the previous studies was prepared by growth of the organism in [$^3$H]glucose and contained a mixture of lipopolysaccharide molecules, some terminating in heptose and some in [$^3$H]glucose. Since different species of lipopolysaccharide enter the monolayer at different rates it is possible that the specific activity of lipopolysaccharide within the film was not the same as the specific activity of the lipopolysaccharide mixture injected into the subphase, resulting in an error in calculation of the number of mol of EI-1 lipopolysaccharide in the film. The possibility also has not been excluded that the surface areas of EI-1 and G30 lipopolysaccharide truly differ. In either case, since the labeled G30 lipopolysaccharide used in the present experiments was grown, labeled, and purified

identically to the unlabeled G30 lipopolysaccharide and since the amount of G30 lipopolysaccharide found in the films was insensitive to isotope dilution, we feel that the molecular areas reported in this study are valid.

What relationship the putative complex has to the active species in the glycosyltransferase reactions remains to be established. It is of interest that the phosphatidylethanolamine/lipopolysaccharide molar ratio giving maximal enzyme activities in the transferase system is approx. 50 : 1 (refs. 13–15, and Fried, V.A. and Rothfield, L.I., unpublished data). This is significantly greater than the 16 : 1 ratio necessary to form the lipopolysaccharide-phosphatidylethanolamine complex. It is possible that the lipopolysaccharide-phosphatidylethanolamine structure is altered by the presence of $Mg^{2+}$ and transferase enzymes in the ultimate functional complex. On the other hand, it is possible that the phosphatidylethanolamine in excess of that necessary to form lipopolysaccharide-phosphatidylethanolamine complex reflects a requirement for a fluid phospholipid matrix in which enzymes and complex can diffuse freely to maximize the turnover number of the reaction.

## Appendix

*Reexamination of assumptions implicit in Eqn. 3*

Eqn. 3 was derived on the assumption the limiting areas of the individual components were unaffected by their interaction in the mixed film. Since we are now postulating the existence of a complex of lipopolysaccharide and phosphatidylethanolamine, it is possible to derive a new expression that accounts for the contribution of the complex to the total area of the film and examine what effect this could have on the interpretation of the stoichiometry shown in Fig. 3. The complete description of this situation is

$$A_T = A_{LPS} \cdot N_{LPS} + A_{PE} \cdot N_{PE} + A_C \cdot N_C \quad (4)$$

where $A_T$ is the total area of the film, $A_{LPS}$, $A_{PE}$, and $A_C$ are the molecular areas of pure lipopolysaccharide, pure phosphatidylethanolamine and complex, respectively, at maximal compression, and $N_{LPS}$, $N_{PE}$ and $N_C$ are the number of molecules of each species present in the film.

It is convenient to define

$$T_{N_{LPS}} = N_{LPS} + N_C \quad (5)$$

$$T_{N_{PE}} = N_{PE} + qN_C \quad (6)$$

where $T_{N_{LPS}}$ and $T_{N_{PE}}$ are total number of molecules of lipopolysaccharide and phosphatidylethanolamine, respectively, in the film, and $q$ is the number of molecules of phosphatidylethanolamine associated with each molecule of lipopolysaccharide within the complex. Rearranging Eqns. 5 and 6 so that $N_{LPS}$ and $N_{PE}$ are expressed as a function of $T_N$ and $N_C$ and substituting into Eqn. 4, we can derive the new expression,

$$A_T = T_{N_{LPS}} \cdot A_{LPS} + T_{N_{PE}} \cdot A_{PE} + N_C \cdot \delta \quad (7)$$

where $\delta$ is the difference between the area of the complex and the area of the sum of its individual parts ($A_C - (A_{LPS} + q \cdot A_{PE})$).

Three cases are possible. These are plotted in Fig. 5 and discussed in the following paragraphs.

(I) The area of the lipopolysaccharide-phosphatidylethanolamine complex is equal to the sum of the individual areas of its components. Here $\delta = 0$ and Eqn. 7 reduces to Eqn. 3,

$$A_T = T_{N_{LPS}} \cdot A_{LPS} + T_{N_{PE}} \cdot A_{PE} \quad (3)$$

This relationship satisfies the experimental result shown in Fig. 2, in that the quantity of lipopolysaccharide in the film is directly proportional to the space available (i.e. the space not occupied by phosphatidylethanolamine molecules) both in the presence and absence of phosphatidylethanolamine.

(II) The area of the complex is greater than the sum of the area of its components. Here $\delta > 0$ and $A_T = T_{N_{LPS}} \cdot A_{LPS} + T_{N_{PE}} \cdot A_{PE} + [\delta] \cdot N_C$. Since $A_T$ is constant and since in the experiments $T_{N_{PE}}$ is the independent variable, the amount of lipopolysaccharide in the film would be less than that predicted by the space available.

(III) The area of the complex is less than the sum of the areas of its separate components. Here $\delta < 0$ and $A_T = T_{N_{LPS}} \cdot A_{LPS} + T_{N_{PE}} \cdot A_{PE} - [\delta] \cdot N_C$. By analogy to case II, more lipopolysaccharide would be found in the film than predicted on the basis of the area not occupied by phosphatidylethanolamine molecules.

In Fig. 5, curves II and III represent the results predicted from a 20% increase or decrease in the relative area of the complex ($A_C$).

Although the data in Fig. 2 cannot rule out minor departures from a linear relationship, inspection of Figs. 2 and 5 indicates that it is unlikely that the complex could have an area different from the sum of its components by greater than ±10%. Thus, it is reasonable to accept the relationship expressed in Eqn. 3 and to conclude that the interactions between lipopolysaccharide and phosphatidylethanolamine which lead to formation of a complex do not lead to a major change in the dimensions of the individual components within the plane of the film.

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