Dissociation and Reassociation of Bacterial Membrane Components

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ABSTRACT The dissociation of the phospholipid, lipopolysaccharide, and protein components of the bacterial cell envelope results in loss of enzyme activities which are normally located in the cell envelope structure and which are involved in lipopolysaccharide biosynthesis. The activity of one of these enzymes, UDP-galactose:lipopolysaccharide α,3 galactosyl transferase, can be restored by the reassociation of purified phospholipid, lipopolysaccharide, and enzyme protein. Reconstitution of activity occurs in stepwise fashion: lipopolysaccharide + phospholipid $\rightarrow$ lipopolysaccharide·phospholipid $\xrightarrow{+\text{ enzyme}}$ enzyme·lipopolysaccharide·phospholipid. The intermediates in the reaction were isolated by gradient centrifugation. The final ternary complex behaves in a similar manner to the intact cell envelope in the enzyme reaction and appears to represent the reconstitution of a portion of the membranous portion of the cell envelope.

Three of the basic questions of membrane biology can be summarized as follows: (*a*) What is the molecular architecture of biological membranes? (*b*) How does this molecular architecture effect and regulate specific membrane functions? (*c*) By what mechanism are individual components assembled to form complete membrane structures? The present discussion will concern itself mainly with the first and second of these questions.

There are two general ways to study membrane structure and function, and both are legitimate. First is the investigation of intact membranes, as illustrated by Dr. Wallach's elegant studies, in which aspects of membrane structure are analyzed by indirect techniques or by direct analysis of whole membranes. The second approach, typified by our own studies and by those of Dr. Racker, involves dissociation of the membrane into its constituents followed by attempts to regain a membrane function by the stepwise reassociation of the separated components.

One group of membrane functions which can be studied in this way are reactions occurring in membranes and controlled by enzymes which are themselves parts of the membrane structure. In recent years several enzyme systems

have been described which are located in biological membranes and whose normal enzyme activity requires the integrity of the membrane structure (for review see reference 1). The subject of my discussion today will be one of these membrane-bound enzyme systems. In this system, as in others, the key to an understanding of the problem lies in the interaction of membrane lipids with proteins and other macromolecules.

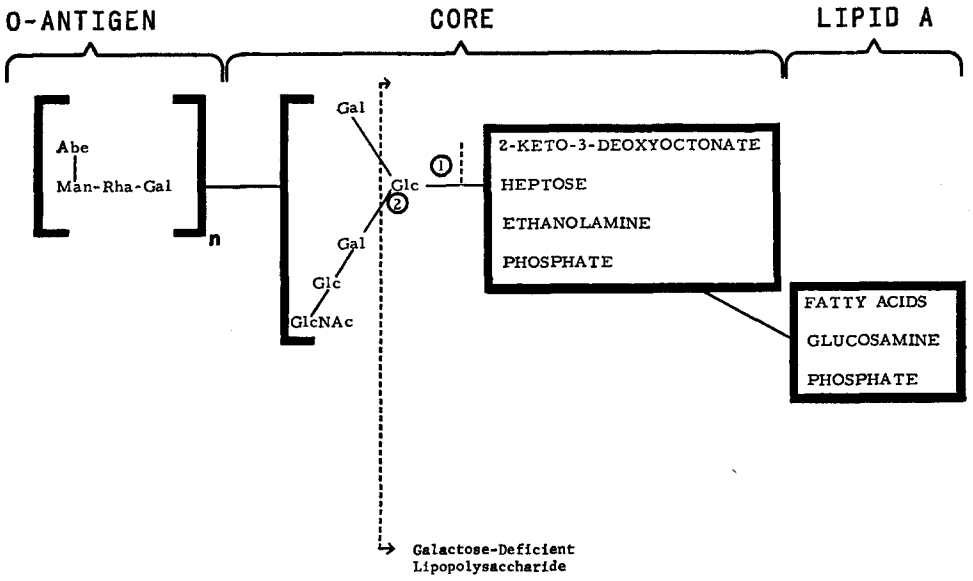

FIGURE 1. Structure of lipopolysaccharide from *S. typhimurium* (2). The numbers refer to the site of action of the glycosyl transferase enzymes involved in biosynthesis of the core polysaccharide. Number 2 refers to UDP-galactose:lipopolysaccharide α,3 galactosyl transferase.

The enzyme proteins to be discussed are located in the cell envelopes of Gram-negative bacteria and are involved in biosynthesis of the endotoxin lipopolysaccharide which is itself a membrane component in these organisms. The lipopolysaccharide is a large molecule consisting of a lipid portion ("Lipid A") in covalent linkage to a complex polysaccharide (Fig. 1). A group of membrane-bound enzymes are involved in the addition of sugar residues during growth of the polysaccharide portion of the molecule (3) and several of these enzymes have been obtained in soluble form from extracts of *Salmonella typhimurium*. The isolated enzymes are inactive when removed from the lipid-rich environment of the cell envelope and the reconstitution of activity requires the specific reassociation of phospholipid, lipopolysaccharide, and enzyme protein. One of these enzymes, UDP-galactose:lipopolysaccharide α,3 galactosyl transferase, has been extensively purified, and the present discussion will focus on this enzyme, which catalyzes the transfer of galactose from UDP-galactose to the core polysaccharide (Fig. 1).

Although the enzyme normally catalyzes the transfer of galactose to lipopolysaccharide, the lipopolysaccharide must remain within the membrane structure of the cell envelope in order to participate as an acceptor in the transfer reaction. Thus, purified lipopolysaccharides were inactive in the reaction catalyzed by the soluble enzyme, although the endogenous lipopolysaccharide of the cell envelope was fully active. This suggested that an additional component of the cell envelope was required, and this proved to be the membrane phospholipids (4).

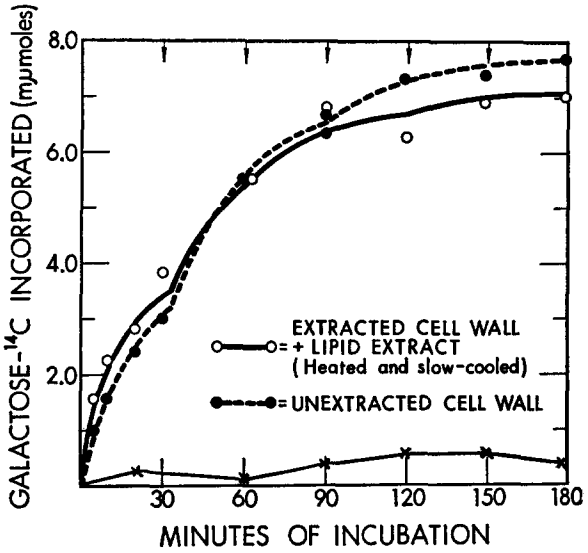

FIGURE 2. Effect of lipid extraction on the galactosyl transferase reaction. The cell envelope fraction was heated to inactivate endogenous transferase activity and was used as acceptor in the transferase reaction both before and after lipid extraction (4). The reaction was performed by adding enzyme and UDP-galactose-^{14}C to the indicated acceptor. The lower curve represents an experiment in which the acceptor was the lipid-extracted cell wall fraction in the absence of added lipid.

TABLE I

REQUIREMENT FOR PHOSPHATIDYL ETHANOLAMINE IN THE GALACTOSYL TRANSFERASE REACTION

The galactosyl transferase reaction was performed as previously described (6), using purified transferase enzyme, but the acceptor was varied as shown. In each case the acceptor was heated to 60°C for 30 min and cooled to room temperature before use.

Acceptor	Galactose incorporation
	mμmoles/10 min
None	0.2
LPS	0.6
PE	0.3
LPS & PE	4.2

TABLE II

EFFECT OF DIFFERENT PHOSPHOLIPIDS ON THE TRANSFERASE REACTION

The indicated phospholipids were substituted for phosphatidyl ethanolamine in the usual reaction mixture (6).

Phosholipid	Enzyme activity
	units/μmole lipid
Dioleyl phosphatidyl ethanolamine	6.35
Phosphatidyl glycerol (*A. tumefacians*)	4.2
β-oleyl-γ-stearoyl phosphatidic acid	6.7
β-oleyl-γ-stearoyl cardiolipin	3.9
β-oleyl-γ-stearoyl phosphatidyl ethanolamine	1.0
Dioleyl phosphatidyl choline	0.01

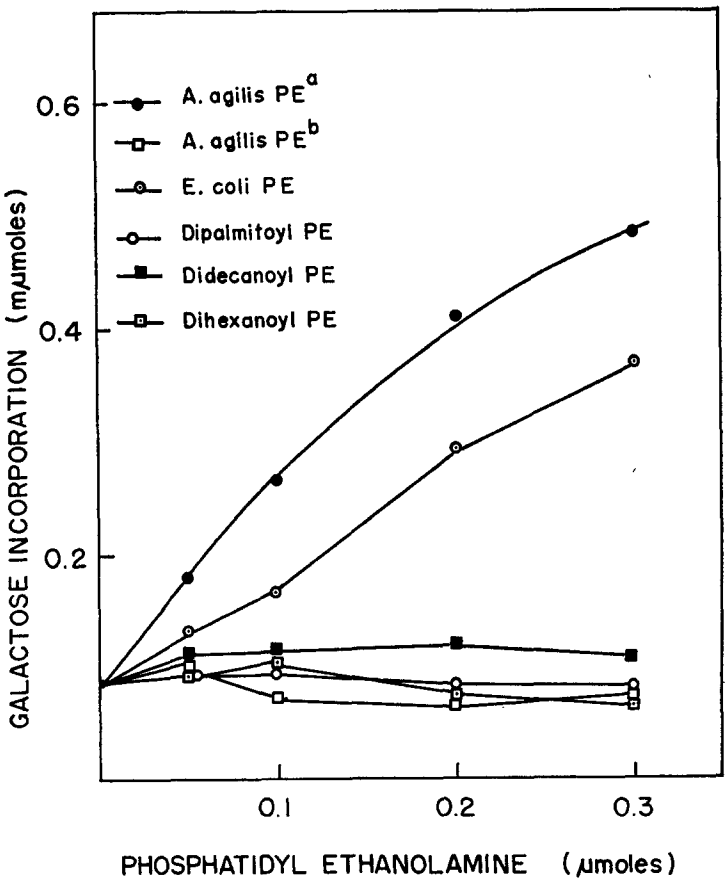

FIGURE 3. Effect of different phosphatidyl ethanolamines on the transferase reaction.[1] *A. agilis PE*[a] refers to native phosphatidyl ethanolamine from *Azotobacter agilis*; *A. agilis PE*[b] refers to *A. agilis PE* that had been subjected to catalytic hydrogenation.

[1] Abbreviations: LPS, lipopolysaccharide; PE, phosphatidyl ethanolamine.

Extraction with lipid solvents was associated with loss of the activity of the cell envelope as an acceptor in the transferase reaction despite the fact that the extraction did not remove the lipopolysaccharide (Fig. 2). Restoration of the acceptor activity was achieved by adding back the lipid extract. The major lipid component in these organisms is phosphatidyl ethanolamine and purified phosphatidyl ethanolamine was also fully effective in restoring the acceptor activity of the lipid-extracted cell envelope.

Reconstitution of the system from the purified components was also possible. There was complete restoration of enzyme activity when purified phospha-

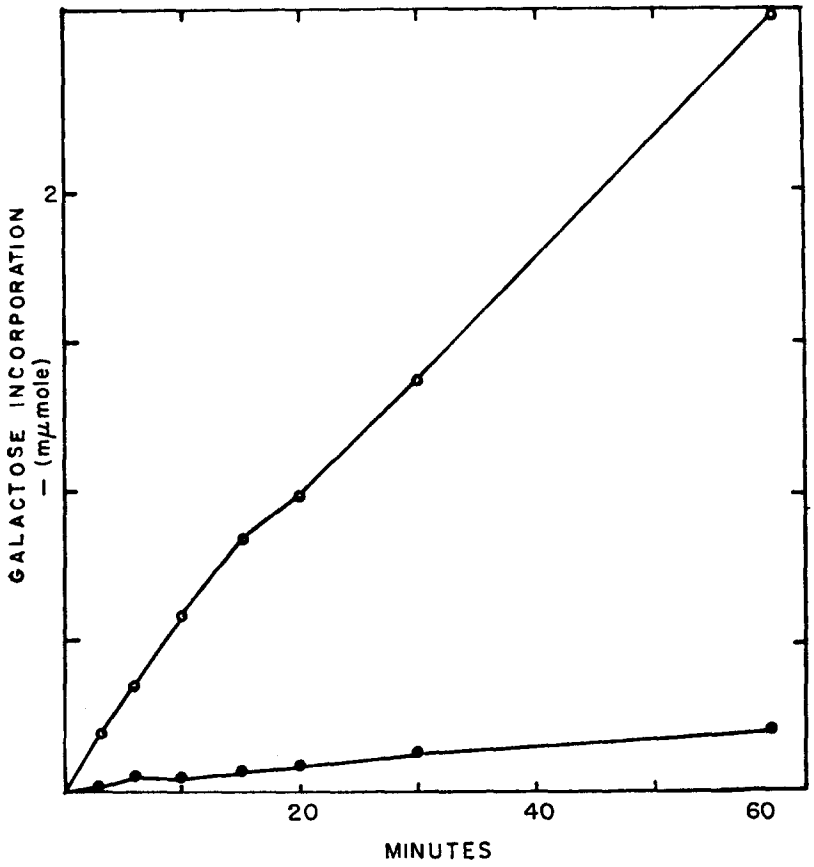

FIGURE 4. Effect of preincubation of lipopolysaccharide and phosphatidyl ethanolamine on the transferase reaction. The upper curve represents an experiment in which lipopolysaccharide and phosphatidyl ethanolamine were mixed, heated to 60°C for 30 min, and cooled to room temperature before the reaction was initiated by addition of enzyme and UDP-galactose-^{14}C. The lower curve describes an experiment in which lipopolysaccharide and phosphatidyl ethanolamine were subjected to the heating procedure separately and were then mixed simultaneously with addition of enzyme and UDP-galactose-^{14}C.

tidyl ethanolamine and lipopolysaccharide were first recombined and the purified enzyme was subsequently added (Table I).

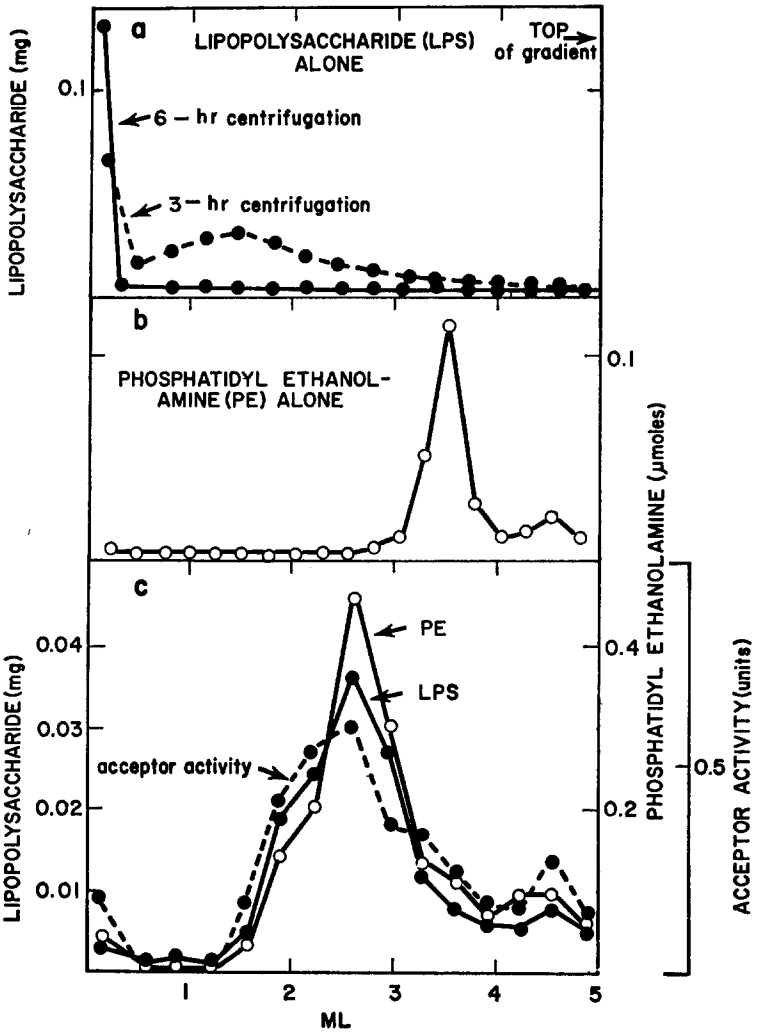

FIGURE 5. Gradient centrifugation of lipopolysaccharide and phosphatidyl ethanolamine. All the components of each gradient were heated together at 60°C and cooled to room temperature before being subjected to centrifugation in a 10–30% sucrose gradient for 18 hr, by which time the equilibrium positions had been reached.

Biological membranes show marked differences in their phospholipid composition, and the present system provided evidence that differences in phospholipid structure are reflected in differences in function (5). Phosphatidyl ethanolamine and several other phospholipids were active in restoring the activity of the transferase system, while phosphatidyl choline, which is absent

from the membranes of these organisms, was completely inactive (Table II). Thus, specificity resides in the polar portion of the phospholipid molecule.

In addition, the fatty acid portion of the molecule also plays a role (Fig. 3). Activity required that the phospholipid contain unsaturated fatty acids,

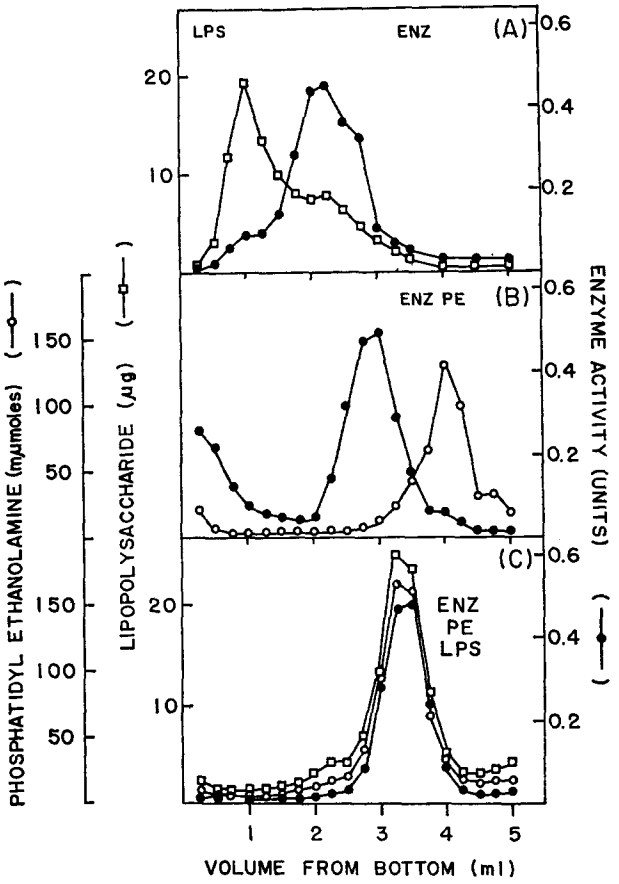

FIGURE 6. Gradient centrifugation of lipopolysaccharide, phosphatidyl ethanolamine, and transferase enzyme. Conditions were as described for Fig. 5 except that equilibrium was not reached in the experiment with enzyme alone.

while no activity was seen with phospholipids in which the fatty acids were saturated. This correlates with the well-established observation that the phospholipids of biological membranes contain large amounts of unsaturated fatty acids.

Reconstitution of the enzyme activity requires the reassociation of three components—phospholipid, lipopolysaccharide, and enzyme protein. The first step involves the interaction of phospholipid and lipopolysaccharide with formation of a phospholipid-lipopolysaccharide complex. In the second step

the enzyme binds to this basic lipid framework, and finally the transfer reaction can be demonstrated by adding the second substrate, UDP-galactose.

The interaction between lipopolysaccharide and phospholipid was most clearly indicated by the requirement that the two components be heated to-

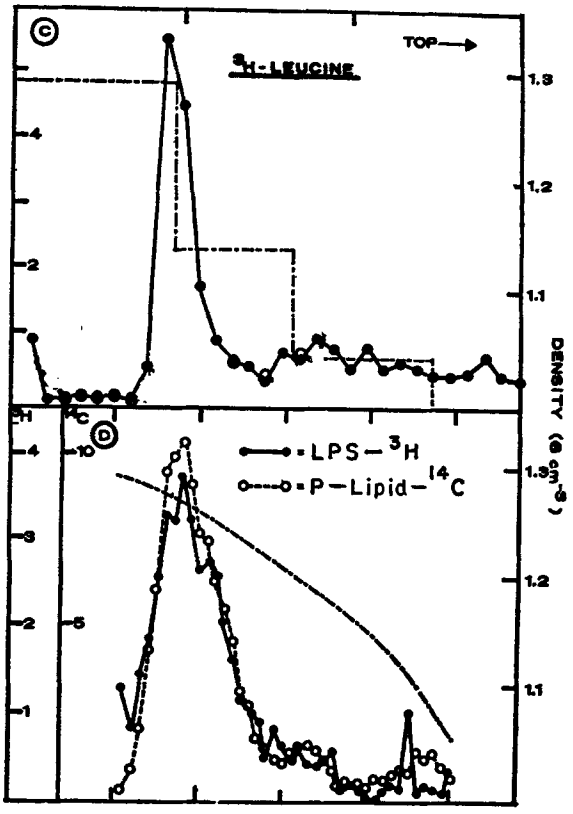

FIGURE 7. Gradient centrifugation of excreted membrane complex. Cells of *E. coli LE-1* were grown in the presence of leucine-^{3}H, to label proteins, galactose-1-^{3}H to label lipopolysaccharide, or oleate-^{14}C to label phospholipids, and were then suspended in complete medium containing chloramphenicol (0.2 mg/ml). After 100 min the culture fluid was collected and subjected to centrifugation in a 30–60% sucrose gradient until equilibrium was reached.

gether prior to adding the transferase enzyme (Fig. 4). As predicted by this mechanism, the optimal concentration of phospholipid for the reaction also varied with the concentration of lipopolysaccharide and was independent of the amount of enzyme.

The binary complex of lipopolysaccharide and phosphatidyl ethanolamine was isolated by density gradient centrifugation (6), taking advantage of the marked difference between the densities of lipopolysaccharide (1.49 g/cc) and phosphatidyl ethanolamine (0.975 g/cc). Centrifugation was performed for

periods of time sufficient to achieve equilibrium as shown by the unchanged positions of the peaks with longer periods of centrifugation. The positions of the components therefore reflect their buoyant densities. When lipopolysaccharide and phosphatidyl ethanolamine were examined separately by this technique the lipopolysaccharide was recovered in a pellet at the bottom of the tube while phosphatidyl ethanolamine was found near the top of the tube. However, when lipopolysaccharide and phosphatidyl ethanolamine were heated together and then subjected to gradient centrifugation, both components were found in a new peak of intermediate density (Fig. 5).

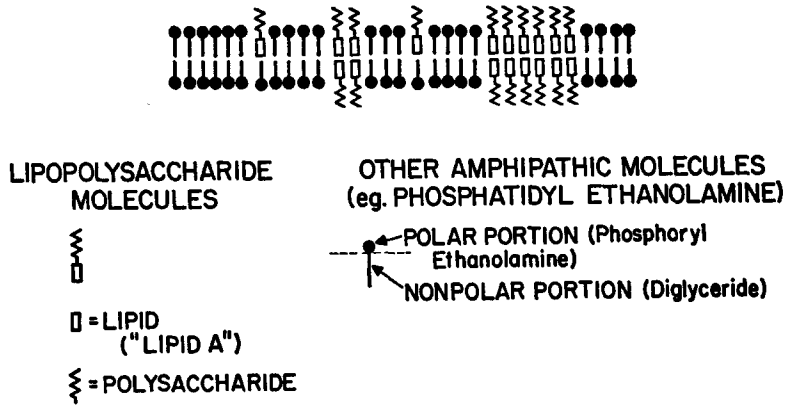

FIGURE 8. Speculative model of the arrangement of lipopolysaccharide and phospholipid molecules in the outer membrane of *E. coli* and *S. typhimurium*.

The gradient centrifugation technique was also used to demonstrate formation of the ternary complex of lipopolysaccharide, phospholipid, and enzyme. When transferase enzyme was added to the lipopolysaccharide-phospholipid complex and gradient centrifugation was then performed, a new peak was observed, containing all three components (Fig. 6). The new peak was fully active in the galactosyl transferase reaction as shown by the rapid transfer of galactose from added UDP-galactose-^{14}C into the lipopolysaccharide. The isolated ternary complex was indistinguishable from the native cell envelope in the kinetics of the transferase reaction. Uridine diphosphate has been identified as the second product of the reaction by thin layer chromatography.

Thus the following sequence of reactions has been demonstrated:

$$1.\ \text{LPS} + \text{PE} \rightarrow \text{LPS}\cdot\text{PE}$$

$$2.\ \text{LPS}\cdot\text{PE} + \text{enzyme} \rightarrow \text{enzyme}\cdot\text{LPS}\cdot\text{PE}$$

$$3.\ \text{Enzyme}\cdot\text{LPS}\cdot\text{PE} + \text{UDP-galactose} \rightarrow \text{galactosyl-LPS}\cdot\text{PE} + \text{UDP} (+ \text{enzyme})$$

Direct evidence that lipopolysaccharide and phosphatidyl ethanolamine are actually present in a common membrane structure at the periphery of the cell envelope has emerged from recent experiments. Segments of the outer membrane of the cell envelope bud off small vesicles into the surrounding medium when protein synthesis is inhibited (7). The lipopolysaccharide, phospholipid, and protein components of the cell envelope were labeled by exposing the growing bacteria to radioactive galactose, oleate, and leucine respectively, and the excreted outer membrane vesicles were then isolated from the culture fluid by density gradient centrifugation to equilibrium as de-

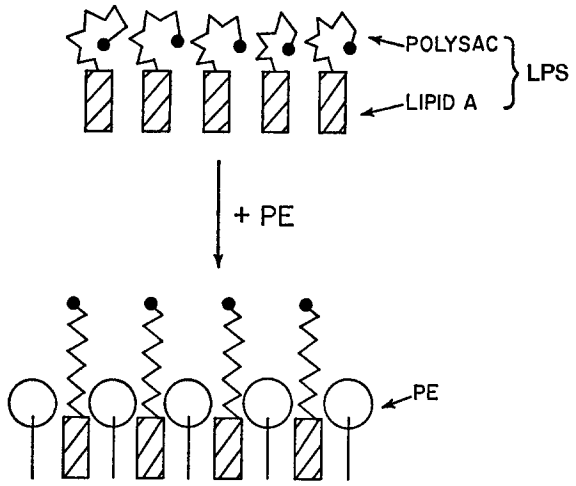

FIGURE 9. One model of the possible effect of phosphatidyl ethanolamine on lipopolysaccharide molecules in the lipopolysaccharide·phosphatidyl ethanolamine complex. The dark ball at the end of the polysaccharide chain represents the terminal glucosyl residue which is the acceptor site for the enzymatic transfer of galactose in the transferase reaction.

scribed above (Fig. 7). All three components were present in a peak whose density corresponded to the peaks prepared from the purified materials (see above). The arrangement of molecules within the in vitro complex is therefore of particular interest because of its probable relevance to the arrangement of these molecules within the outer membrane of the cell envelope.

An acceptable model of the reconstituted membrane complex should be consistent with the evidence that lipopolysaccharide and phosphatidyl ethanolamine molecules are closely associated within a common structure (Fig. 7) and that this association probably involves interactions between both the polar (phosphorylethanolamine and polysaccharide) and nonpolar portions of the molecules (Table II, Fig. 3). Such a speculative model is shown in Fig. 8, in which lipopolysaccharide and phospholipid molecules lie side by side within a common leaflet structure.

The interaction of lipopolysaccharide with phospholipid appears to make polysaccharide chains accessible to the enzyme as shown by the inefficient binding of enzyme to lipopolysaccharide in the absence of phospholipid and by the five-fold greater yield when the reaction was permitted to go to completion in the presence of phosphatidyl ethanolamine as compared with the reaction in its absence. This suggests that the interpolation of molecules of phospholipid may change the conformation of the adjacent lipopolysaccharide molecules so that the terminal polysaccharide region is made accessible to the enzyme, as schematically illustrated in Fig. 9. Proof for this hypothesis is not yet available, nor is evidence at hand to define the details of the interaction of the enzyme protein with the lipopolysaccharide-phospholipid complex.

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REFERENCES

1. ROTHFIELD, L. and A. FINKELSTEIN. 1968. Membrane biochemistry. *Ann. Rev. Biochem.* **37**:463.
2. LÜDERITZ, O., A. M. STAUB, and O. WESTPHAL. 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. *Bacteriol. Rev.* **30**:192.
3. OSBORN, M. J., S. M. ROSEN, L. ROTHFIELD, L. ZELEZNICK, and B. L. HORECKER. 1964. Lipopolysaccharide of the gram-negative cell wall. *Science.* **145**:783.
4. ROTHFIELD, L., and B. L. HORECKER. 1964. The role of cell-wall lipid in the biosynthesis of bacterial lipopolysaccharide. *Proc. Nat. Acad. Sci. U.S.A.* **52**:939.
5. ROTHFIELD, L., and M. PEARLMAN. 1966. The role of cell envelope phospholipid in the enzymatic synthesis of bacterial lipopolysaccharide: structural requirements of the phospholipid molecule. *J. Biol. Chem.* **241**:1386.
6. WEISER, M. M., and L. ROTHFIELD. 1968. The reassociation of lipopolysaccharide, phospholipid and transferase enzymes of the bacterial cell envelope: isolation of binary and ternary complexes. *J. Biol. Chem.* **243**:1320.
7. KNOX, K., M. VESK, and E. WORK. 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *E. coli*. *J. Bacteriol.* **92**:1206.