The Role of Cell Envelope Phospholipid in the Enzymatic Synthesis of Bacterial Lipopolysaccharide

STRUCTURAL REQUIREMENTS OF THE PHOSPHOLIPID MOLECULE*

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SUMMARY

The role of phospholipid in the uridine diphosphate-galactose:lipopolysaccharide galactosyltransferase reaction has been studied in extracts of Salmonella typhimurium by utilizing synthetic and natural phospholipids and phospholipids altered by venom digestion, alkaline hydrolysis, and catalytic hydrogenation. Activity was seen with derivatives of phosphatic acid containing two unsaturated or cyclopropane fatty acids, and either a hydroxyl group, ethanolamine, glycerol, or polyglycerol phosphate (in cardiolipin) as the component attached to the α-phosphate group. No activity was seen with phosphatidylcholine.

The lipopolysaccharides of gram-negative enteric bacteria are located in the cell envelope and are responsible for the O-antigenic specificity and the endotoxin activity of these organisms (1). The cell envelope also contains a large amount of lipid, primarily phospholipid, with smaller amounts of free fatty acids, coenzyme Q, and other trace components (2). It has previously been shown (3) that the lipid fraction of the cell envelope participates in at least two of the enzyme-catalyzed reactions in the biosynthesis of the lipopolysaccharide of Salmonella typhimurium.

Glucose-deficient lipopolysaccharide +

UDP-glucose + lipid + MgCl₂ + soluble enzyme →

 UDP-glucose + lipid + MgCl₂ + soluble enzyme →

Galactose-deficient lipopolysaccharide +

UDP-galactose + lipid + MgCl₂ + soluble enzyme →

Galactose-deficient lipopolysaccharide +

Reactions 1 and 2 are catalyzed by uridine diphosphate-glucose:lipopolysaccharide glucosyltransferase and uridine diphosphate-galactose:lipopolysaccharide galactosyltransferase, respectively (4). The acceptor lipopolysaccharide for Reaction 1 is isolated from a mutant unable to synthesize UDP-glucose. For Reaction 2 the acceptor lipopolysaccharide is obtained from a mutant unable to synthesize UDP-galactose. No activity is seen with purified lipopolysaccharides as acceptors unless a phospholipid fraction from the cell envelope is added, and both reactions require that the lipid and lipopolysaccharide be combined under specific conditions of heating and slow cooling before the lipopolysaccharide can act as an acceptor for sugar transfer (3). Heating and slow cooling appear necessary to permit an interaction to occur between lipopolysaccharide and lipid, since no acceptor activity is seen when the two components are heated and slowly cooled separately. It has also been shown that the soluble transferase enzymes bind to the lipopolysaccharide-lipid complex produced by heating and slow cooling (5). The reaction sequence can be outlined as follows:

Lipopolysaccharide + lipid →

lipopolysaccharide-lipid + enzyme + MgCl₂ + nucleotide sugar

glycosyllipopolysaccharide-lipid (+ enzyme)

The present communication is concerned with the structure of lipids which participate in these reactions. We have previously reported that the crude lipid fractions obtained from the cell envelopes of a variety of microorganisms were active in the enzymatic transferase reactions and phosphatidylethanolamine has been identified as the active lipid component in S. typhimurium, Escherichia coli, and Azotobacter agilis (3).

Studies with synthetic lipids have now confirmed that phosphatidylethanolamine is fully active in the transferase reactions. Other phospholipids have also shown activity, and it has been established that the structural requirements for activity reside in the nature of the fatty acid residues and in the component attached to the phosphate group.

In these studies, the galactosyltransferase reaction was studied because of the greater activity and stability of the soluble galactosyltransferase enzyme.

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† Career Investigator of the Health Research Council of the City of New York.
Materials and Analytic Procedures—Galactose-deficient lipopolysaccharide was isolated and purified from *S. typhimurium* G-80 (deficient in UDP-galactose-4-epimerase) as previously described (6). The enzyme was the 106,000 X g supernatant fraction prepared from sonic extracts of *S. typhimurium* EI-1 (deficient in UDP-galactose-4 epimerase and phosphoglucoisomerase) (4). In a few instances, a 10- to 20-fold purified enzyme preparation was prepared from the same source. UDP-galactose-36C randomly labeled in the galactose residue was prepared as previously described (6) or was purchased from the International Chemical and Nuclear Corporation. Hephaestus and protein were determined by the methods of Dische (7) and Lowry et al. (8), respectively, and lipid concentrations were determined by measuring total phosphate (9), in the case of phospholipids, or by dry weights. Ethanolamine was determined by the dinitrofluorobenzene method (10). Detergents were obtained from the following sources: hexadecyltrimethylammonium bromide, Fluka A. G.; oleyl acid phosphate, Hooker Chemical Corporation; polyoxyethylene sorbitan (Tween 80) and sodium dodecyl sulfate, Sigma; dioleyl phosphoric acid, Kirschell Laboratories; Cutsem, Fisher Scientific Company; dodecyl pyridinium chloride, a gift from Dr. John Law.

**Chromatography**—Thin layer chromatography was performed on Silica Gel G (Brinkman) with the following solvents: Solvent 1, chloroform-methanol-water (65:25:4); Solvent 2, benzene-pyridine-water (60:60:11); Solvent 3, chloroform-methanol-water (65:25:8). When preparative thin layer chromatography was performed, the plates were sprayed with 0.01% 2,7-dichlorofluorescein. The spots were visualized with iodine and visualized by spraying with 2,7-dichlorofluorescein. The bands corresponding to phosphatidylethanolamine, phosphatidyl-N-methyl-ethanolamine, and phosphatidylcholine were eluted with methanol and with chloroform-methanol (3:1) and were rechromatographed in Solvent 2, which permitted separation of phosphatidylethanolamine and phosphatidyl-N-methyl-ethanolamine. After elution, samples of the purified lipids were hydrolyzed in 2 N HCl in evacuated tubes at 100° for 12 hours, followed by ether extraction. The aqueous phases were chromatographed on Whatman No. 1 paper (pre-treated with 1 N KCl) in the solvent system of Bremer, Figard, and Greenberg (15). The spots were visualized with iodine and ninhydrin spray and were compared with authentic ethanolamine, ethanolamine phosphate, N-methyl ethanolamine, N,N-dimethyl ethanolamine, and sodium dodecyl sulfate, Sigma; dioleyl phosphoric acid, Kirschell Laboratories; Cutsem, Fisher Scientific Company; dodecyl pyridinium chloride, a gift from Dr. John Law.

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**Isolation of Phospholipids**—The lipids of *E. coli*, *S. typhimurium*, and *Clostridium butyricum* were extracted by mixing the centrifuged cells with 5 volumes of chloroform-methanol (3:1, v/v) at room temperature for 60 min. After filtration, the residue was reextracted in a similar manner. The chloroform-methanol extracts were pooled, washed twice by the method of Folch, Lees, and Sloane Stanley (11), and taken to dryness under reduced pressure. The material was dissolved in a minimal volume of chloroform and chromatographed on silicic acid columns (Bio-Rad), with 10 mg of lipid per g of silicic acid, at 10–15°. Fatty acids and neutral lipids were eluted with chloroform, and successive elution with mixtures of chloroform-methanol (9:1 → 5:1 → 3:1 → 1:1) was performed with 5 bed volumes of each mixture. Fractions of 5 ml each were collected and were characterized by thin layer chromatography with Solvent 1 together with authentic lipid standards, visualizing the spots with iodine vapor. The fractions containing phosphatidylethanolamine were pooled and rechromatographed on a silicic acid column in an identical manner. After the second chromatography, those fractions were selected which showed only a single chromatographic spot corresponding to authentic phosphatidylethanolamine when 0.1 mg of lipid was examined in the thin layer chromatographic system.

In the extract from *C. butyricum*, the contaminating plasmalogens were removed and the phosphatidylethanolamine was separated from phosphatidyl-N-methyl-ethanolamine by preparative thin layer chromatography as described by Hildebrand and Law (12). Ratios of ethanolamine to phosphate of 0.9:1.3 were found in the purified phosphatidylethanolamine fractions prepared from the three organisms. The purified lipids were precipitated with acetone and lyophilized from benzene prior to use.

Phospholipids of *Neurospora crassa* were isolated from crude lipid extract of mutant strain 47904, which was generously sent to us by Dr. J. Nyc of the University of California at Los Angeles. This mutant strain accumulates large amounts of phosphatidylethanolamine, phosphatidyl-N-methyl-ethanolamine, and phosphatidyl-N,N-dimethyl-ethanolamine (13). These compounds were isolated by silicic acid column chromatography as described by Hall and Nyc (14), except that the peak containing phosphatidyl-N,N-methyl-ethanolamine and phosphatidylethanolamine was eluted in 10% methanol in chloroform, while the peak containing phosphatidyl-N,N-dimethyl-ethanolamine (contaminated with a small amount of phosphatidylethanolamine) was eluted in 15% methanol in chloroform. The compounds were further purified by preparative thin layer chromatography in Solvent 1 and visualized by spraying with 2,7-dichlorofluorescein. After elution, samples of the purified lipids were hydrolyzed in 2 N HCl in evacuated tubes at 100° for 12 hours, followed by ether extraction. The aqueous phases were chromatographed on Whatman No. 1 paper (pre-treated with 1 N KCl) in the solvent system of Bremer, Figard, and Greenberg (15). The spots were visualized with iodine and ninhydrin spray and were compared with authentic ethanolamine, ethanolamine phosphate, N-methyl ethanolamine, N,N-dimethyl ethanolamine, and sodium dodecyl sulfate, Sigma; dioleyl phosphoric acid, Kirschell Laboratories; Cutsem, Fisher Scientific Company; dodecyl pyridinium chloride, a gift from Dr. John Law.

**Other Lipids**—Phosphatidylethanolamine from *Aerobacter agilis*, phosphatidylglycerol from *Aerobacter tumefaciens*, synthetic 1,3,9,10-methyleneoctadecanoic acid, and synthetic dihexanoyl- and didecanoylphosphatidylethanolamine were gifts from Dr. John Law of Harvard University. Synthetic β-oleyl-γ-stearoyl-l-α-phosphatidylethanolamine, β-oleyl-γ-stearoyl-l-α-phosphatidic acid, and glycerocephosphoryl ethanolamine were gifts from Dr. Erich Baer of The University of Toronto. Synthetic dioleyl-1-α-phosphatidylethanolamine, dioleyl-1-α-phosphatidylcholine, β-oleyl-γ-stearoylcardiolipin, and β-oleyl-γ-stearoyl-1-alanyldiposphatidylglycerol were generous gifts from Dr. L. van Deenen of The Rijksuniversiteit Te Utrecht. Dioleyl-glycerol (mixed α and β isomers), synthetic dipalmitoyl-l-α-phosphatidylethanolamine, synthetic dipalmitoyl-l-α-phosphatidylcholine, oleic acid, palmitic acid, and l-α-glycerylphosphorylcholine were purchased from Sigma. Purified phrenosin, kerasin, and sphingomyelin (beef brain) were gifts from Dr. W. Norton of this institution, and a mixture of ethanolamine phosphatides from beef heart (60% plasmalogens and 40% diacyl) was a gift from Dr. M. Rapport also of this institution. All of the lipids were examined by thin layer chromatography prior to use.
When necessary they were purified by preparative thin layer chromatography until only a single spot was seen.

Assays The assay for activity of UDP-galactose:lipopolysaccharide galactosyltransferase was based on measurement of the incorporation of galactose-\(^{14}\)C into acid-insoluble material.

Galactose-deficient lipopolysaccharide (containing 0.07 \(\mu\)mole of heptose in 0.05 ml of 50 mM Tris-HCl, pH 8.5) was mixed with lipid (0.05 ml of methanol), and the mixture was heated to 60°C for 30 min and then gradually cooled to 23°C over a 2-hour period. The remaining components were then added, so that the final assay mixture contained lipopolysaccharide, lipid, 50 mM Tris-HCl buffer, pH 8.5, 8 mM MgCl\(_2\), 0.08 mM UDP-galactose-\(^{14}\)C (1600 cpm per \(\mu\)mole), and enzyme (0.25 mg of protein) in a total volume of 0.25 ml.

After incubation for 10 min at 37°C, 2 \(\mu\)l of 5\% trichloroacetic acid were added, the precipitate was separated by centrifugation and washed twice with 2 \(\mu\)l of 5\% trichloroacetic acid, the final precipitate was suspended in 50% ethanol-0.5\% concentrated NH\(_3\), and the radioactivity was determined in a windowless gas flow counter. It has previously been shown (3) that this represents incorporation into lipopolysaccharide. Zero time controls (acid added before enzyme) and controls lacking lipopolysaccharide and lipid always showed less than 70 cpm above background in this assay. At this concentration, methanol itself produced no stimulation or inhibition of the enzyme reaction. The results are expressed as units of activity (1 unit = 1 \(\mu\)mole of galactose-\(^{14}\)C incorporated in 10 min).

**RESULTS**

**Characteristics of System.**—In the complete system, the rate of galactose incorporation was proportional to the concentration of dioleylphosphatidylethanolamine from 0.04 to 0.64 \(\mu\)mole per ml, and maximal incorporation was seen with concentrations above 1.2 \(\mu\)mole per ml (Fig. 1). Other active lipids showed a similar relation of lipid concentration to enzyme activity, but the degree of activity, expressed as units of enzyme activity per \(\mu\)mole of lipid, varied considerably, depending on the lipid being tested. At higher concentrations of lipid, the enzyme activity reached a plateau. This maximal level of enzyme activity was similar for most of the active lipids tested, although with lipids of low activity, the plateau region was not reached at the concentration of lipid tested. At high concentrations of lipids there was some times inhibition of the enzyme reaction. For a quantitative comparison of different lipids, activity was therefore measured in the linear portion of the concentration curve and was expressed as units of enzyme activity per \(\mu\)mole of lipid. The absolute values varied in different experiments due to differences in the enzyme preparations.

The initial studies were concerned with phosphatidylethanolamine since it was previously shown that this phospholipid is responsible for the activity of the lipid fraction of the cell envelope in most bacterial strains tested (3).

**Fatty Acids.**—The presence of the fatty acid portion of the phosphatidylethanolamine molecule was essential for activity. When both fatty acid residues were removed by alkaline hydrolysis (Table I), the fatty acids and glycerophosphorylethanolamine resulting from this treatment showed negligible activity in the galactosyltransferase system.

For full activity, two unsaturated fatty acid residues in the...
phosphatidylethanolamine molecule were required (Fig. 1 and Table II). Thus, synthetic dioleophosphatidylethanolamine showed marked activity. When 1 oleic acid residue was replaced by a saturated fatty acid (β-oleyl-γ-stearoylphosphatidylethanolamine), a considerably lower activity was seen, and phosphatidyl-

ethanolamines in which both fatty acids were saturated (dihexanoyl-, didecanoyl-, and dipalmitoylphosphatidylethanolamines) showed only slight activity.

The essential role of the unsaturated fatty acid residues was confirmed by catalytic hydrogenation of the phosphatidylethanolamine isolated from Azotobacter agilis. In the native phospholipid, 65% of the fatty acid residues are unsaturated (12). Hydrogenation was carried to completion and resulted in loss of approximately 90% of the original activity of the phospholipid (Table II). To control other nonspecific effects of the hydrogenation procedure, phosphatidylethanolamine isolated from E. coli grown to stationary phase was subjected to the same hydrogenation conditions. The E. coli phosphatidylethanolamine contained cyclopropane fatty acids in place of unsaturated acids (12), so that no reduction of fatty acids could occur, and no loss of activity was seen.

In stationary phase cells of several bacterial strains, the unsaturated fatty acid residues found in actively dividing cultures are converted to cyclopropane fatty acids, in which a cyclopropane group replaces the double bond (2, 17, 18). The activity of phosphatidylethanolamines containing cyclopropane fatty acids was generally similar to that seen with unsaturated fatty acids (Table II). In the phosphatidylethanolamines of A. agilis and E. coli the unsaturated or cyclopropane fatty acids are predominantly (65 to 80%) in the β position, while in C. butyricum they are largely (70%) in the γ position (12). The compounds isolated from all three species were active in the galactosyltransferase system (Table II), indicating that no significant specificity lies in the position of the unsaturated or cyclopropane fatty acid residue. The activity of the bacterial phospholipids cannot be directly compared with the synthetic dioleoyl- and β-oleyl-γ-stearoylphosphatidylethanolamines, since the fatty acids differ and since the bacterial phosphatidylethanolamines are probably heterogeneous, consisting of mixtures of molecules containing one or two saturated fatty acids, or none at all.

Lysophosphatidylethanolamines—When the β-fatty acid residues were removed by enzymatic hydrolysis, the resulting monocyclic (lyso) derivatives lost most of the activity seen with the original compounds. The nature of the remaining fatty acid residue had no effect on the degree of residual activity. Thus, γ-monoacylphosphatidylethanolamines were prepared by treatment of various phosphatidylethanolamines with venom of Ancistrodon piscivorus piscivorus. This venom has been shown by Lands to cleave specifically the β-acyl ester bond of phosphatidylethanolamine. By varying the phosphatidylethanolamine substrate, lysophosphatidylethanolamines were prepared in which the remaining fatty acid residues were wholly unsaturated (prepared from dioleophosphatidylethanolamine), largely cyclopropane acids (prepared from C. butyricum phosphatidylethanolamine, in which 66% of the γ-fatty acids are cyclopropane

TABLE II

<table>
<thead>
<tr>
<th>Source of phosphatidylethanolamine</th>
<th>Fatty acid components</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>Un saturated (dioleyl)</td>
<td>6.35</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Mixed (β-oleyl-γ-stearoyl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Saturated (dihexanoyl)</td>
<td>0.01</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Saturated (didecanoyl)</td>
<td>0.09</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Saturated (dipalmitoyl)</td>
<td>0.08</td>
</tr>
<tr>
<td>A. agilis</td>
<td>β, 85% unsaturated; γ, 16% unsaturated</td>
<td>11.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>β, 87%, Δ2, Δ7, γ, 21% Δ6</td>
<td>5.8</td>
</tr>
<tr>
<td>C. butyricum</td>
<td>β, 28%, Δ2, Δ7, 66% Δ6</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* Δ = cyclopropane acids.

TABLE III

Catalytic hydrogenation of phosphatidylethanolamine

Hydrogenation was performed by adding the purified phosphatidylethanolamine (2.5 mg in 3 ml of methanol) to 0 mg of PtO2 in 2.5 ml of methanol, after first exposing the PtO2 suspension to H2 for 15 min. The mixture was then exposed to H2 gas at atmospheric pressure and room temperature for 15 min, until no further uptake of H2 was seen. After centrifugation, the PtO2 was washed twice with chloroform methanol (8:1), and the washings were added to the methanol supernatant. The supernatant was evaporated to dryness under reduced pressure and was then dissolved in 0.5 ml of methanol. Aliquots containing 0.05, 0.1, and 0.25 μmole of phosphate were assayed in the galactosyltransferase reaction. Thin layer chromatography in Solvent 1 before and after the hydrogenation procedure showed a slight change in mobility of the A. agilis phosphatidylethanolamine, but no appearance of any new components. There was no visible change in the thin layer chromatographic pattern of the E. coli phosphatidylethanolamine after hydrogenation. When lipid was completely omitted from the assay procedure, there was 0.13 unit of activity per assay tube.

Lipid | Enzyme activity (units/μmole lipid)
--- | ---
A. agilis phosphatidylethanolamine | Before hydrogenation 13.5 After hydrogenation 1.8
E. coli phosphatidylethanolamine | Before hydrogenation 6.6 After hydrogenation 7.7

* The hydrogenation experiments were performed with the generous assistance of Dr. Alemka Kisic of this institution.

acids), or largely saturated (prepared from E. coli phosphatidylethanolamine, in which 79% of the γ-fatty acids are saturated) (12). In all of the cases, the monoacyl derivative showed relatively slight activity when compared with the parent compound (Table IV). The fatty acids resulting from the enzymatic hydrolyses were resolated and assayed and also showed slight ac-

Dr. W. E. Lands of the University of Michigan (personal communication) has tested A. piscivorus piscivorus venom, with previously described techniques (19), and has shown that it specifically hydrolyses the β-fatty ester linkage of phosphatidylethanolamine.
E. coli phosphatidylethanolamine

Dioleylphosphatidylethanolamine

fluorescein, with mobilities identical to oleic acid (Band I),

was subjected to preparative thin layer chromatography in the

system. Three bands were visualized with 2,7-dichloro-

recovered, compared with 2.7 mg of lipid in the original reaction

by thin layer chromatography in Solvent 1, visualizing the spots

with iodine vapor. At completion, the reaction was stopped by

at 15 min intervals and the reaction was followed

mixture. Samples containing 0.05, 0.11, and 0.2 rmole of each

alanylphosphatidylglycerol was more active than E. coli phosphatidylethanolamine.

It is significant that \(\beta\)-oleyl-\(\gamma\)-palmitoylphosphatic acid was considerably more active than \(\beta\)-oleyl-\(\gamma\)-palmitoylphosphatidylethanolamine.

Phosphatidylcholines were completely inactive in the trans-

ferase system, at all concentration levels. Synthetic phospha-
tidylcholines were tested in which both fatty acid residues were

unsaturated (dioleyl) or saturated (dipalmitoyl). In addition,
native phosphatidylcholine isolated from beef brain (containing

large amounts of highly unsaturated fatty acids) and from Neuro-

spora crassa were also without detectable activity, even when

tested at levels as high as 1.2 \(\mu\)moles of lipid per ml. The

phosphatidylcholines did not inhibit the activity of phosphatidyl-

choline when both lipids were present in the same reaction

mixture.

The choline residue differs from ethanolamine in the presence of three \(N\)-methyl groups and in the resulting positive charge. These structural characteristics were further studied by isolating phosphatidylethanolamine, phosphatidyl-\(N\)-methylethanolamine, phosphatidyl-\(N\),\(N\)-dime-
thylethanolamine, and phospha-
tidylcholine from a lipid extract of Neurospora crassa strain

47904. These compounds are thought to occur as successive

intermediates in the biosynthesis of phosphatidylcholine in

Neurospora (14). When assayed under the usual conditions at

pH 8.5 to 9.0, there was progressively less activity with increasing
degrees of methylation of the amino group (Table VI). These

compounds were also assayed at higher pH values where only

choline retains a positive charge. Above pH 10, the activities of

phosphatidylethanolamine, phosphatidyl-\(N\)-methylethanol-
amine, and phosphatidyl-\(N\),\(N\)-dimethylethanolamine were simi-

lar, while phosphatidylcholine remained essentially inactive.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Enzyme activity (units/(\mu)mole lipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioleylphosphatidylethanolamine</td>
<td>6.35</td>
</tr>
<tr>
<td>(\beta)-Oleyl-(\gamma)-stearoyl phosphatic acid</td>
<td>6.7</td>
</tr>
<tr>
<td>Phosphatidylglycerol (A. tumefaciens)</td>
<td>4.2</td>
</tr>
<tr>
<td>(\beta)-Oleyl-(\gamma)-stearoyl cardiolipin</td>
<td>3.9</td>
</tr>
</tbody>
</table>
| \(\beta\)-Oleyl-\(\gamma\)-stearoylphosphatidylethanol-
amine | 1.0                                    |
| Phosphatidylserine (beef brain) | 1.0                                    |
| Phosphatidylcholine     |                                        |
| Dioleyl                 | 0.01                                   |
| Dipalmitoyl            | 0.32                                   |
| Beef brain             | <0.01                                  |
| N. crassa              | 0.02                                   |
| Dioleylglycerol        | 0.01                                   |
| Oleic acid             | 0.03                                   |
| Palmitic acid          | <0.01                                  |
| Cyclopropane acid\(a\) | 0.64                                   |
| Kerasin                | 1.66                                   |
| Phrenosin              | <0.01                                  |
| Phrenosin              | <0.01                                  |
| Sphingomyelmin         | <0.01                                  |
| Ethanolamine phosphatides\(a\) | 8.2                                    |

\(a\) 9,10-Methylene octadecanoic acid.

\(a\) Approximately 60% plasmalogen and 40% diacyl phospha-
tidylethanolamine.
TABLE VI
Activity of methylated phosphatidylethanolamine

<table>
<thead>
<tr>
<th>pH</th>
<th>Phosphatidylethanolamine</th>
<th>Phosphatidyl-N,N-dimethylethanolamine</th>
<th>Phosphatidyl-N,N-dimethyl-N,N-trimethyl-10.5</th>
<th>Phosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>0.45</td>
<td>0.40</td>
<td>0.38</td>
<td>0.04</td>
</tr>
<tr>
<td>9.0</td>
<td>0.52</td>
<td>0.45</td>
<td>0.54</td>
<td>0.08</td>
</tr>
<tr>
<td>8.5</td>
<td>0.25</td>
<td>0.55</td>
<td>0.45</td>
<td>0.04</td>
</tr>
<tr>
<td>7.5</td>
<td>0.48</td>
<td>0.57</td>
<td>0.29</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The differences in activity of the methylated phosphatidylethanolamines in the galactosyltransferase system at different pH values could not be ascribed to difference in surface charge of the phospholipids. Microelectrophoresis was performed by the method of Bangham et al. (20), permitting direct measurement of the mobility of lipid particles in an electric field (Fig. 2). The mobilities of phosphatidylethanolamine, phosphatidyl-N-methyl-N,N dimethylethanolamine, and phosphatidyl-N,N,N,N tetramethylethanolamine were similar between pH 6.5 and 8.5 (minus 3.3 to minus 3.6 μsec⁻¹ volt⁻¹ cm⁻²). Above pH 9.0, the three phosphatidyls all acquired a greater negative surface potential, presumably owing to deprotonation of the amino groups. There was no correlation between mobility and activity in the enzyme system.

The apparent pKₐ values of ethanolamine, N-methylethanolamine, and N,N-dimethylethanolamine were determined by direct titration, and were 9.4, 9.3, and 9.05, respectively.

The basic α-glycerophosphate backbone was necessary for activity, since dioleoylglycerol was inactive. The specificity of phospholipids in the galactosyltransferase reaction resides in both the polar and nonpolar portions of the lipid molecule. Unsataturated and cyclopropane fatty acids are both effective in conferring activity on phospholipids while saturated fatty acids are inactive. The activity of cyclopropane acids eliminates the possibility that the unsaturated fatty acids play a role in the reaction by virtue of the electronic configuration or chemical reactivity of the double bond. Phospholipids containing unsaturated or cyclopropane acids are also similar in their solubility characteristics in polar solvents and in their tendencies to form micellar dispersions. Thus, these compounds readily formed stable water-soluble dispersions, when treated by the

DISCUSSION

The specificity of phospholipids in the galactosyltransferase reaction resides in both the polar and nonpolar portions of the lipid molecule. Unsaturation and cyclopropane fatty acids are both effective in conferring activity on phospholipids while saturated fatty acids are ineffective. The activity of cyclopropane acids eliminates the possibility that the unsaturated fatty acids play a role in the reaction by virtue of the electronic configuration or chemical reactivity of the double bond. Phospholipids containing unsaturated or cyclopropane acids are also similar in their solubility characteristics in polar solvents and in their tendencies to form micellar dispersions. Thus, these compounds readily formed stable water-soluble dispersions, when treated by the
technique of Fleischer and Klouwen (21), and they were soluble in methanol. We have been unable to produce stable micellar dispersions of phosphatidylethanolamines containing saturated fatty acids of similar chain length either by the Fleischer and Klouwen technique or by sonic disintegration, and the saturated compounds are quite insoluble in methanol. It is likely, therefore, that the explanation for the activity of phospholipids which contain unsaturated and cyclopropane acids lies in their ability to form a required micellar or liquid crystalline structure when exposed to a polar environment.

The complete absence of activity with phosphatidylcholine demonstrates that the polar portion of the lipid molecule also plays an important role in the lipid-lipopolysaccharide interaction, possibly by interacting with the polar portion of the lipopolysaccharide. If lipopolysaccharides and phospholipid were both present in a common micellar or liquid crystalline structure, such polar group interactions would be expected.

The similarity in activity of phosphatidylethanolamine, phosphatidyl-N-methylethanolamine, and phosphatidyl-N,N-dimethylethanolamine at high pH and the absence of activity of phosphatidylethanolamine (which retains the positive charge of the choline residue) suggest that the polar portion of the lipid must possess a negative charge in order to be fully active. The 6-fold higher activity of β-oleyl-γ-stearoyl phosphatidic acid than the corresponding phosphatidylethanolamine supports this view. However, the differences in activity of phosphatidylethanolamine and its mono and dimethyl derivatives at pH 7.5 to 9.0 cannot be explained on this basis. The electrophoretic studies demonstrate that the surface charge of the three phospholipids is almost identical, and the titration and electrophoretic data both indicate that there is no significant difference in the degree of protonation of the amino groups at pH 7.5 to 9.0. In addition, there was no correlation of enzyme activity with negative surface charge when detergents were added to alter the surface potential of the phospholipids. It is therefore unlikely that the observed differences of enzyme activity in this pH range are due to differences in charge of the phospholipids. It is probable that other effects of the methyl groups are responsible, and steric factors seem most likely. The galactosyltransferase system thereby differs from the phospholipase systems studied by Bangham and Dawson (22), in which enzyme activity was clearly correlated with the surface charge of the phospholipid substrates.

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