Studies of a Phospholipid-Requiring Bacterial Enzyme. II. The Role of Phospholipid in the Uridine Diphosphate Galactose:Lipopolysaccharide α-3-Galactosyl Transferase Reaction*

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ABSTRACT: Phospholipid is required as a cofactor in the reaction catalyzed by uridine diphosphate galactose:lipopolysaccharide α-3-galactosyl transferase. The role of phospholipid was studied, using an extensively purified preparation of the enzyme.

The technique of equilibrium density gradient centrifugation was used to determine the ability of different phospholipids to form binary complexes with lipopolysaccharide or with enzyme. Certain phospholipids formed binary complexes with lipopolysaccharide but not with enzyme; others formed binary complexes with enzyme but not with lipopolysaccharide. Both of these types of phospholipid were inactive in the enzyme system. In contrast, phospholipids which were able to form binary complexes both with lipopolysaccharide and with enzyme were active. The data suggest a model in which the enzyme reaction involves binding of phospholipid to both the lipopolysaccharide substrate and to the enzyme protein.

The preceding paper (Endo and Rothfield, 1969) described the purification and properties of UDP-galactose:lipopolysaccharide α-3-galactosyl transferase. This enzyme, isolated from *Salmonella typhimurium*, catalyzes

$$\text{UDP-galactose} + \text{galactose-deficient lipopolysaccharide} \xrightarrow{\text{phospholipid}} \text{galactosyl lipopolysaccharide} + \text{UDP}$$

The system is of particular interest because phospholipid is required as a cofactor in the reaction. The present paper describes detailed studies of the role of phospholipids in this reaction, leading to formulation of a tentative mechanism for the observed effects.

Materials and Methods

UDP-galactose:lipopolysaccharide α-3-galactosyl transferase was purified from extracts of *S. typhimurium* G-30A by purification method A as described in the preceding paper and the DEAE-cellulose fraction (fraction VI, specific activity 15,000 units/mg of protein) was used unless otherwise specified. Lipopolysaccharides were purified by phenol extraction and Mg^{2+} precipitation (Osborn *et al.*, 1962). Glucose content of galactose-deficient G-30 lipopolysaccharide was calculated from the heptose concentration, using the molar ratio of glucose–heptose (1:2) which had been determined in parallel experiments. For preparation of soluble polysaccharide and lipid A the lipopolysaccharide was suspended in 0.01 N HCl to a concentration of 0.5 mg/ml and the suspension was heated at 100° for 45 min. The residue was collected by centrifugation and was subjected twice more to the same hydrolysis procedure. The pooled supernatant fractions ("soluble polysaccharide") and the final residue ("lipid A") were evaporated to dryness repeatedly before use. Radioactive galactose-deficient lipopolysaccharide containing glucose-1-^{3}H as the nonreducing terminal glucose residue[1] was prepared by growth of *S. typhimurium EI-1* in the presence of glucose-1-^{3}H (Weiser and Rothfield, 1968) and was mixed with nonradioactive galactose-deficient lipopolysaccharide from *S. typhimurium* G-30 to reach the desired specific activity. Radioactive lipopolysaccharide of wild-type *S. typhimurium* was prepared by growing strain G-30 in the presence of galactose-1-^{14}C.

Phosphatidylethanolamine from *Azotobacter agilis* was prepared as described previously (Weiser and Rothfield, 1968). Hydrogenated *A. agilis* phosphatidylethanolamine was isolated by silicic acid column chromatography following catalytic reduction (Rothfield and Pearlman, 1966). Synthetic dipalmitoyl-L-α-phosphatidylethanolamine was obtained from the Sigma Chemical Co., St. Louis, and didecanoyl- and dihexanoylphosphatidylethanolamine were prepared by the method of Maurukas *et al.* (1963) and were gifts from Dr. John Law.

Density gradient centrifugation was performed as described in the preceding paper except that centrifugation was continued until there was no further change in the positions of

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[1] The structures of the lipopolysaccharides used in these studies are indicated in Figure 1 of the preceding paper (Endo and Rothfield, 1969).

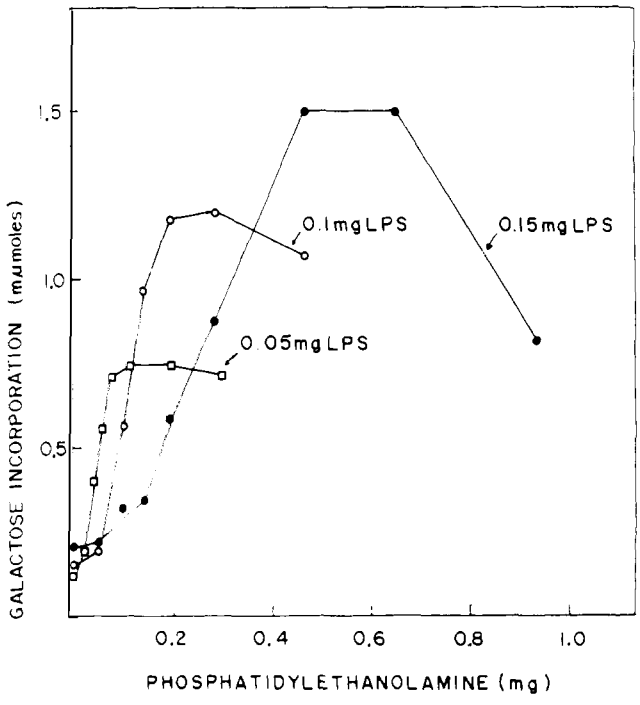

FIGURE 1: Effect of the concentration of phosphatidylethanolamine (PE) on the rate of the galactosyl transferase reaction. Assays (Endo and Rothfield, 1969) were performed for 10 min using 1 unit of enzyme. The content of *A. agilis* phosphatidylethanolamine and of G-30 lipopolysaccharide was varied, and the figure indicates the amount in each 0.25-ml final reaction mixture.

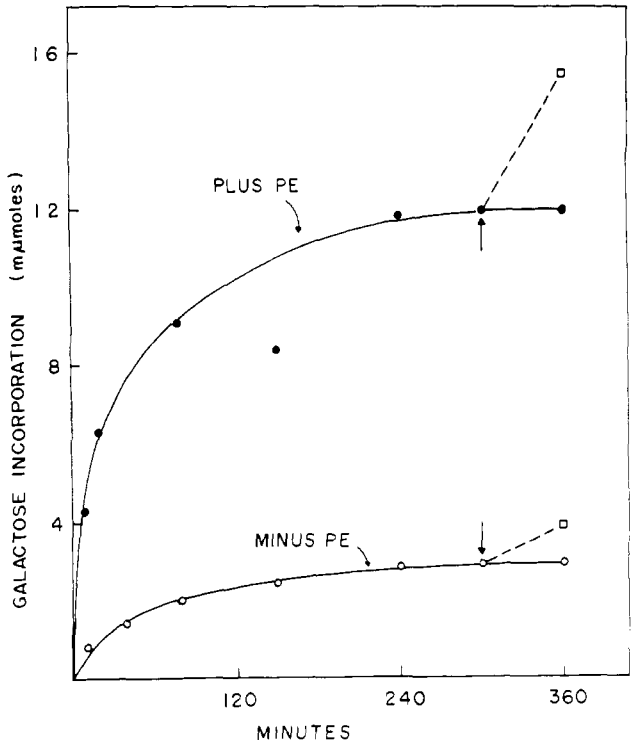

FIGURE 2: Effect of phosphatidylethanolamine on the time course of the galactosyl transferase reaction. Incubation mixtures were prepared in the usual manner, using 5 units of enzyme. The periods of incubation were varied as indicated in the figure and galactose incorporation was determined in the standard assay procedure. The lower curve indicates experiments in which phosphatidylethanolamine was omitted. The arrows indicate the time of addition of an additional 0.07 mg of lipopolysaccharide to the tubes indicated by the interrupted lines.

lipopolysaccharides and phospholipids. The positions of these components therefore reflect differences in their buoyant densities in the sucrose solution.

Results

Effects of Phosphatidylethanolamine on the Course of the Enzyme Reaction. A low but measurable level of enzyme activity was seen in the absence of phospholipid, and with increasing amounts of phosphatidylethanolamine there was a progressive increase in the initial rate of the reaction (Figure 1). The magnitude of the activation by phosphatidylethanolamine was a function of the ratio of phosphatidylethanolamine to lipopolysaccharide rather than the absolute concentration of phosphatidylethanolamine. In each case a maximal reaction rate was seen when the ratio of phosphatidylethanolamine to lipopolysaccharide was approximately 3:1 (mg/mg). This is consistent with the previous suggestion that the major role of phospholipid in the reaction is related to its interaction with lipopolysaccharide rather than to a direct effect on the enzyme.

In the presence of phosphatidylethanolamine the reaction continued for several hours with a final incorporation of approximately 0.6 residue of galactose for each glucose residue in the acceptor lipopolysaccharide (Figure 2), or 60% of the theoretical maximal yield (Endo and Rothfield, 1969). A yield of 0.4–0.6 residue of galactose/glucose residue was also obtained when widely different concentrations of lipopolysaccharide were assayed. Termination of the reaction appeared to be due to exhaustion of accessible acceptor sites since addition of fresh acceptor lipopolysaccharide caused a resumption of the reaction. Addition of fresh enzyme or UDP-galactose-^{14}C after the reaction had come to a halt did not cause resumption of the reaction and did not change the final yield. In a separate experiment the assay mixture was subjected to the heating and slow-cooling procedure after completion of the reaction. Fresh enzyme was then added to determine whether additional acceptor sites were made available, but this resulted in no further incorporation of galactose.

In the absence of phosphatidylethanolamine, both the rate and the yield of the reaction were considerably lower than in the presence of optimal amounts of the phospholipid (Figure 2). The V_{max} was 13-fold higher in the presence of phosphatidylethanolamine than in its absence (Figure 3) and the yield was increased approximately fourfold (Figure 2).

An interaction between phosphatidylethanolamine and lipopolysaccharide appeared to be an obligatory first step in the reaction. When phosphatidylethanolamine and lipopolysaccharide were preincubated, the subsequent addition of enzyme and UDP-galactose caused a prompt initiation of the reaction (Figure 4). In contrast, when the preincubation step was omitted the rate of the reaction was negligible. The temperature required for the interaction between lipopolysaccharide and phosphatidylethanolamine varied with different preparations

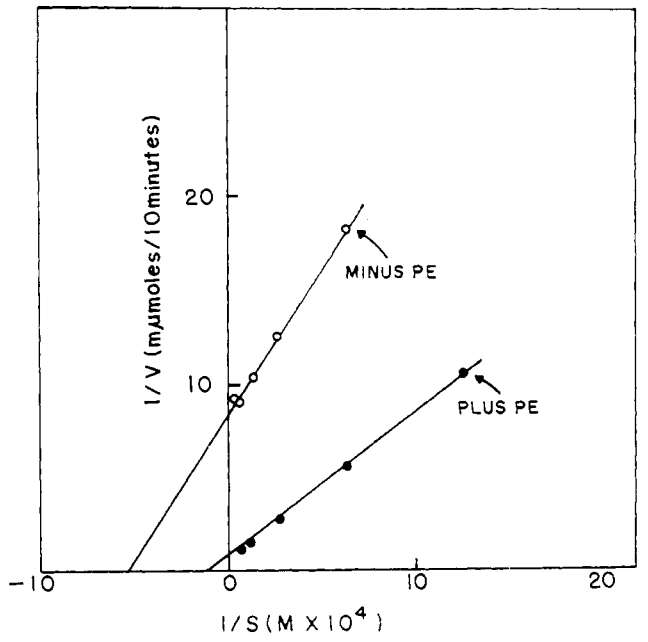

FIGURE 3: Effect of phosphatidylethanolamine on the double-reciprocal plot of reaction velocity *vs.* concentration of lipopolysaccharide in the galactosyl transferase reaction. Assays were performed with 1.5 units of enzyme. The concentration of G-30 lipopolysaccharide is expressed in terms of heptose concentration. In the upper curve, phosphatidylethanolamine was omitted from the reaction mixture. In the lower curve, the concentration of *A. agilis* phosphatidylethanolamine was 0.25 μmole/ml of final reaction mixture.

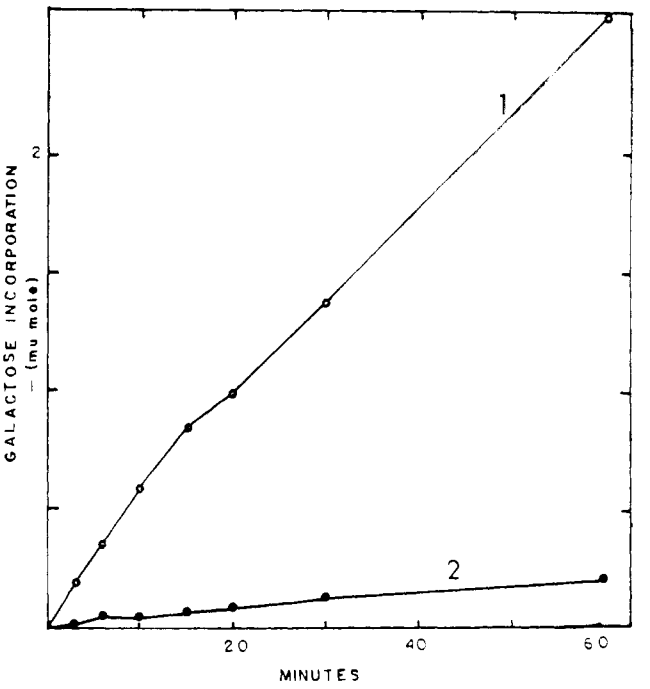

FIGURE 4: Effect of preincubation of lipopolysaccharide and phosphatidylethanolamine. (1) G-30 lipopolysaccharide (0.78 mg) and *A. agilis* phosphatidylethanolamine (2.1 mg in 0.2 ml of methanol) were mixed in 1.2 ml of 0.2 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA. The suspension was heated at 60° for 30 min and was allowed to cool to room temperature before use. The remaining components of the reaction were then added (0.15 ml of 2 mM UDP-galactose-^{14}C, 6000 cpm/mμmole, 0.15 ml of 0.1 M $MgCl_2$, 7 units of enzyme, and water to a total volume of 2.6 ml). The mixture was incubated at 24° and 0.25-ml samples were removed at intervals for determination of trichloracetic acid precipitable radioactivity. (2) G-30 lipopolysaccharide (0.78 mg) was suspended in 0.7 ml of 0.2 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA; *A. agilis* phosphatidylethanolamine (2.1 mg in 0.2 ml of methanol) was suspended in 0.5 ml of the same Tris-EDTA buffer. The two suspensions were heated separately to 60° and cooled to room temperature as described, and were then mixed. Enzyme, UDP-galactose-^{14}C, and $MgCl_2$ were added, and incubation at 24° was performed as described above.

of lipopolysaccharide, but in most cases, preincubation at 37° for 10 min was sufficient to produce a fully active acceptor.

Effects of Changes in Fatty Acid Residues. Our previous studies had shown that the unpurified enzyme was active only in the presence of phospholipids containing unsaturated or cyclopropane fatty acids (Rothfield and Pearlman, 1966). This was confirmed in the present study of the purified enzyme (Figure 5). Full activity was seen with phosphatidylethanolamine from *A. agilis*, which contains approximately 60% unsaturated fatty acids, and with *E. coli* phosphatidylethanolamine, which contains a large proportion of cyclopropane fatty acids.[2] Hydrogenation of *A. agilis* phosphatidylethanolamine caused complete loss of activity, and no activity was seen with three phosphatidylethanolamines containing saturated fatty acids (dipalmitoyl-, didecanoyl-, and dihexanoylphosphatidylethanolamine).

Mixtures of didecanoylphosphatidylethanolamine and dipalmitoylphosphatidylethanolamine were tested at a variety of concentrations and were unable to substitute for phosphatidylethanolamine from *A. agilis* in the transferase reaction.

Inhibition by Didecanoylphosphatidylethanolamine. In studying the effects of phosphatidylethanolamines containing saturated fatty acids, it was found that didecanoylphosphatidylethanolamine was an effective inhibitor of the enzyme reaction. Didecanoylphosphatidylethanolamine causes a marked decrease in both the rate and yield of the reaction (Figure 6). There was no effect on the V_{max} (Figure 7).

Lipopolysaccharide–Phospholipid Interactions. Gradient centrifugation experiments provided evidence that the interaction of phosphatidylethanolamine with lipopolysaccharide was required for both activation (*A. agilis* phosphatidylethanolamine) and inhibition (didecanoylphosphatidylethanolamine) of the enzyme reaction. Lipopolysaccharide–phosphatidylethanolamine complexes were identified by subjecting mixtures of the two compounds to centrifugation in sucrose gradients until equilibrium was reached (Figure 8). The positions of the components therefore reflected their buoyant densities.

When lipopolysaccharide and phosphatidylethanolamine were examined separately by this technique the lipopolysaccharide was recovered in a pellet at the bottom of the tube while phosphatidylethanolamine remained in the upper half of the gradient. On the other hand, when gradient centrifugation was performed on mixtures of lipopolysaccharide and either *A.*

[2] Analyses of fatty acid components of these phospholipids have been published (Rothfield and Pearlman, 1966).

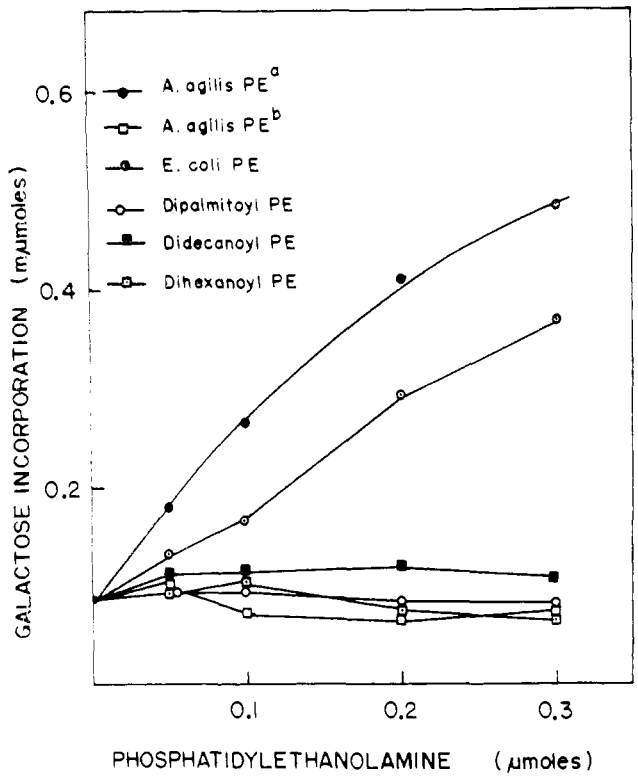

FIGURE 5: Effect of different phosphatidylethanolamines on the transferase reaction. Assays were carried out in the usual manner except that the type of phosphatidylethanolamine was varied as indicated in the figure. *A. agilis* PE[a] refers to native phosphatidylethanolamine. *A. agilis* PE[b] refers to phosphatidylethanolamine that had been subjected to catalytic hydrogenation. In each case the lipopolysaccharide–phospholipid mixture was heated and slow-cooled prior to the addition of enzyme and UDP-galactose-^{14}C (Endo and Rothfield, 1969).

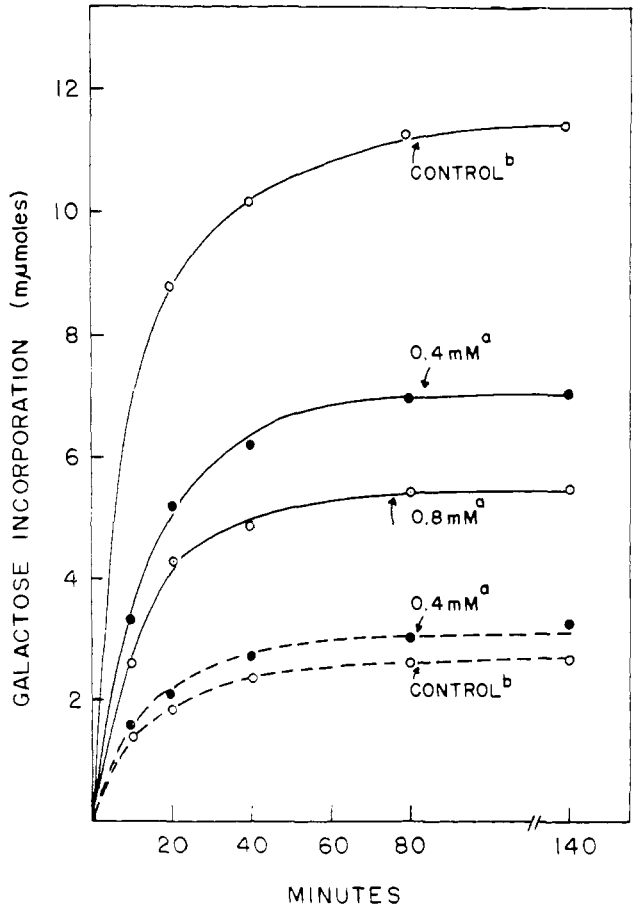

FIGURE 6: Inhibition of the galactosyl transferase reaction by didecanoylphosphatidylethanolamine: time course. Assays were performed in the usual manner using 7 units of enzyme and the time of incubation was varied as shown. Where indicated didecanoylphosphatidylethanolamine was added to the acceptor mixture before the 60° heating step. The lower two curves (●— — —●) indicate experiments in which *A. agilis* phosphatidylethanolamine was omitted from the acceptor mixture; in the upper three curves the acceptor mixture contained the usual amounts of G-30 lipopolysaccharide and *A. agilis* phosphatidylethanolamine. (a) The concentration of didecanoylphosphatidylethanolamine in the final reaction mixture is indicated in the figure. (b) Didecanoylphosphatidylethanolamine was omitted.

agilis or didecanoylphosphatidylethanolamine (Figure 8a,b), lipopolysaccharide and phosphatidylethanolamine were both recovered in a common peak within the gradient, indicating formation of binary complexes of the two components. In contrast, dipalmitoylphosphatidylethanolamine and hydrogenated *A. agilis* phosphatidylethanolamine were neither activators nor inhibitors of the reaction and did not form significant amounts of binary complex with lipopolysaccharide (Figure 8c,d).

Enzyme–Phospholid Interactions. In previous studies with a crude enzyme preparation, galactosyl transferase activity was shown to bind efficiently only to lipopolysaccharide–phosphatidylethanolamine complexes, with little binding of enzyme to phosphatidylethanolamine or lipopolysaccharide alone. In contrast, the purified enzyme formed complexes with a variety of different lipids in the absence of lipopolysaccharide. In addition to formation of a complex between enzyme and *A. agilis* phosphatidylethanolamine (Figure 9a), enzyme–phospholipid complexes were also seen with *E. coli* and dipalmitoylphosphatidylethanolamine and with hydrogenated *A. agilis* phosphatidylethanolamine. No complex was formed between enzyme and either didecanoyl- (Figure 9b) or dihexanoylphosphatidylethanolamine. Addition of egg phosphatidylcholine resulted in aggregation of the enzyme as indicated by the appearance of multiple rapidly sedimenting peaks in the sucrose gradient.

Enzyme–Lipopolysaccharide Interactions. The gradient centrifugation technique was also used to study the interaction between enzyme and lipopolysaccharide. An interaction of enzyme and lipopolysaccharide was indicated by the presence of a portion of the lipopolysaccharide in a small peak together with most of the enzyme activity (Figure 10a); the bulk of the lipopolysaccharide was present in a second peak in the lower 20% of the tube. In contrast, lipopolysaccharide was completely recovered in the pellet fraction when sedimented in the absence of enzyme. Similar results were obtained with mixtures of enzyme and a variety of other lipopolysaccharides (G-30F, G-30G, SL1032, TV119, and LT2). There was no evidence of enzyme binding to the polysaccharide portion of the molecule (Figure 10b), while the addition of lipid A appeared

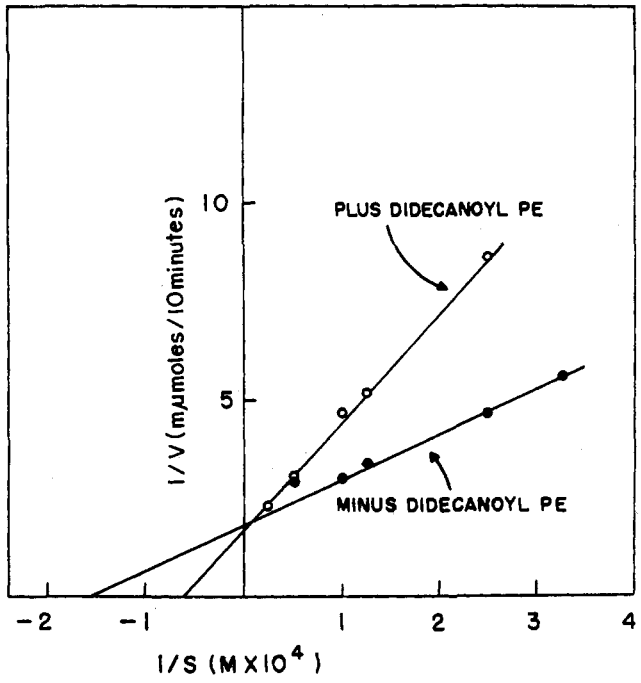

FIGURE 7: Inhibition of the galactosyl transferase reaction by didecanoylphosphatidylethanolamine: Lineweaver–Burk plot. Assays were performed as in Figure 3. The upper curve (—○—) indicates reactions in which didecanoylphosphatidylethanolamine was added to the acceptor mixture prior to the 60° heating step to give a final concentration of 0.1 μmole/0.25 ml final assay mixture.

to cause aggregation of the enzyme (Figure 10c) without loss of enzyme activity.

Enzyme–Lipopolysaccharide–Phospholipid Interactions. Ternary complexes of enzyme, lipopolysaccharide, and phosphatidylethanolamine were readily demonstrated with *A. agilis* and *E. coli* phosphatidylethanolamine, and with didecanoylphosphatidylethanolamine (Figure 11a,b). In contrast, no ternary complex was formed when dipalmitoyl- or hydrogenated *A. agilis* phosphatidylethanolamine was studied (Figure 11c). The limitations of the technique do not permit accurate measurements of possible differences in affinity of the enzyme for lipopolysaccharide–phospholipid complexes containing different phospholipids (*e.g.* didecanoyl- *vs. A. agilis* phosphatidylethanolamine).

It had been noted previously that no binding of enzyme to phospholipids was seen when crude enzyme preparations were studied (Weiser and Rothfield, 1968). Since enzyme binding to lipopolysaccharide–phospholipid complexes was readily demonstrable, this suggested that components of the crude enzyme preparation may inhibit formation of nonspecific enzyme–lipid complexes while not preventing formation of the active ternary complex of enzyme, lipopolysaccharide, and phosphatidylethanolamine. In keeping with this view, addition of a small amount of crude extract (identical with the amount used in previous studies) (Weiser and Rothfield, 1968) to mixtures of purified enzyme and phospholipid prevented formation of enzyme–phospholipid complexes (Figure 12b). On the other hand, the crude extract did not prevent binding of the enzyme to the lipopolysaccharide–phosphatidylethanol-

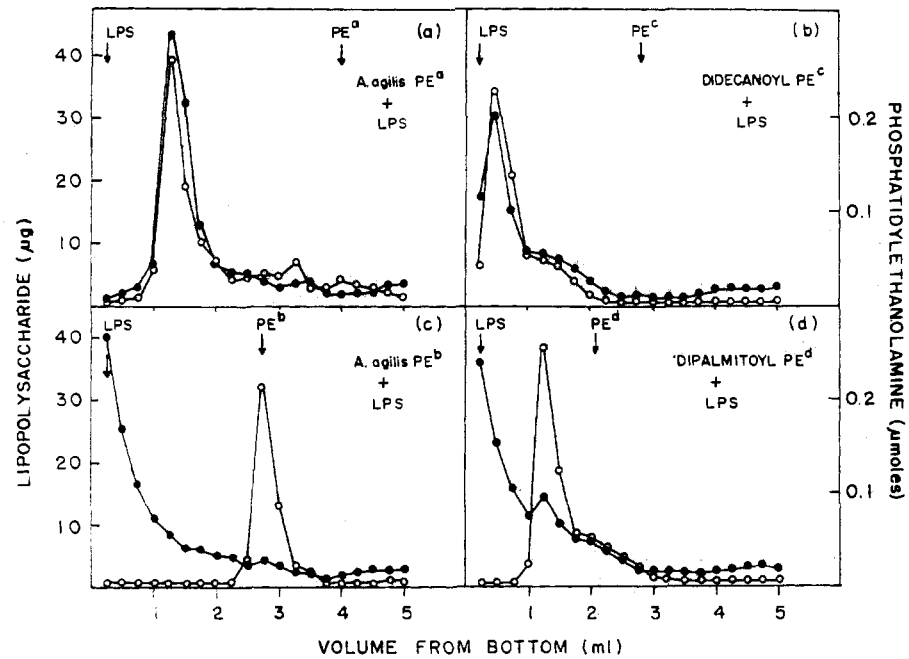

FIGURE 8: Gradient centrifugation of mixtures of lipopolysaccharide and phosphatidylethanolamine. Mixtures of 1.0 mg of phosphatidylethanolamine in 0.1 ml of methanol (PE) and 0.3 mg of ^{3}H-labeled G-30 lipopolysaccharide (5000 cpm) in 0.2 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA were heated at 60° for 30 min and then cooled slowly. Gradient centrifugation was performed for 16 hr and fractions of 0.25 ml were collected. PE^a and PE^b refer to *A. agilis* phosphatidylethanolamine-^{14}C and to hydrogenated *A. agilis* phosphatidylethanolamine-^{14}C, respectively. PE^c and PE^d represent didecanoyl- and dipalmitoylphosphatidylethanolamine; 0.1-ml samples of each fraction were used for analysis. The amounts of lipopolysaccharide and of *A. agilis* phosphatidylethanolamine were determined by liquid scintillation counting; didecanoyl- and dipalmitoylethanolamine were determined by the ninhydrin method (Colowick and Kaplan, 1957). The arrows in the upper portions of the figures indicate the positions of lipopolysaccharide (LPS) and phosphatidylethanolamine when subjected to gradient centrifugation individually in parallel experiments. (—●—) Lipopolysaccharide and (—○—) phosphatidylethanolamine.

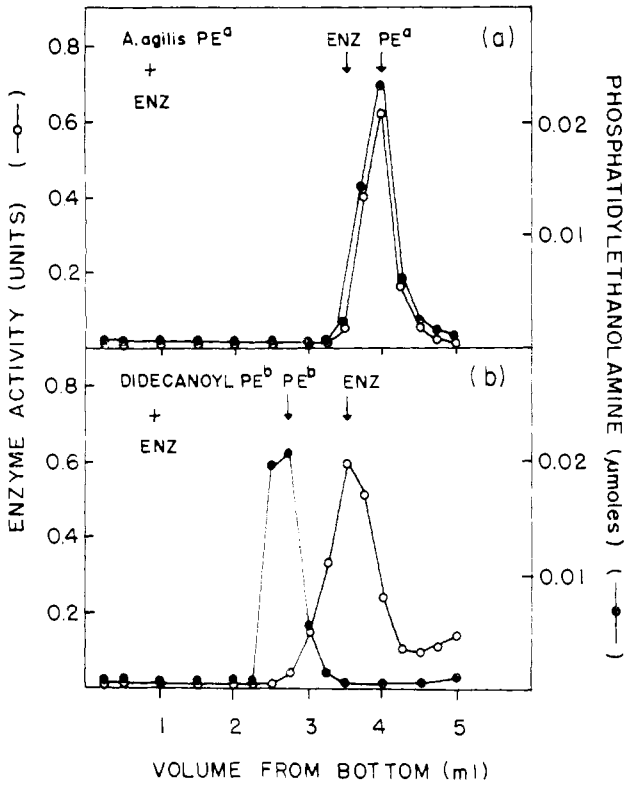

FIGURE 9: Gradient centrifugation of mixtures of purified enzyme and phosphatidylethanolamine. Mixtures of 5 μg (70 units) of purified enzyme and 0.1 mg of PE in 0.4 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA were subjected to gradient centrifugation for 16 hr. Enzyme activity was determined by the standard assay procedure, using 0.01-ml samples of each 0.25-ml fraction. The arrows at the top of the figure show the positions of enzyme (ENZ) and phosphatidylethanolamine when they were subjected to gradient centrifugation separately. PE[a] and PE[b] refer to *A. agilis* and didecanoylphosphatidylethanolamine, respectively. Approximately 60–70% of the total enzyme activity applied to the gradient was recovered in each experiment.

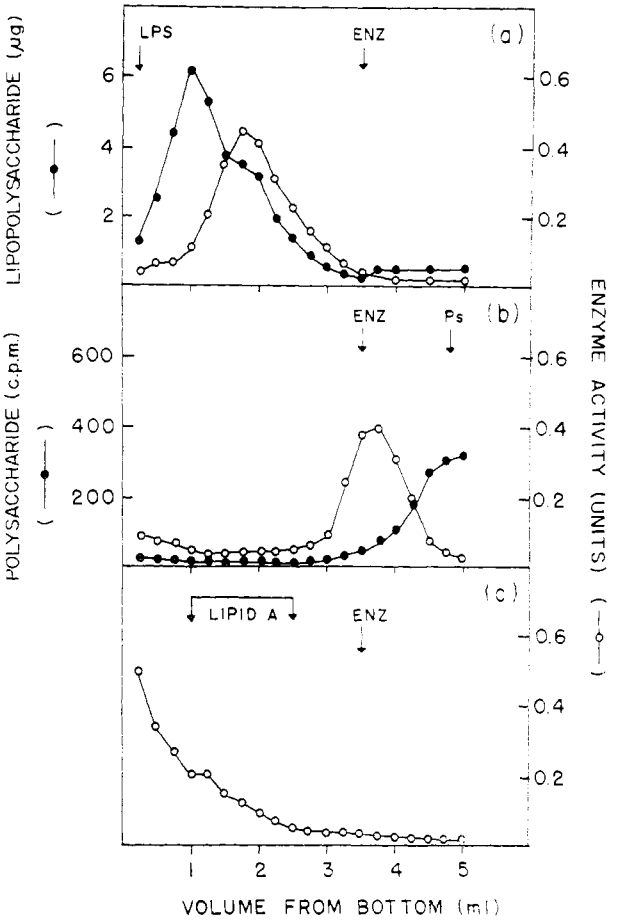

FIGURE 10: Gradient centrifugation of mixtures of enzyme and lipopolysaccharide. (a) A mixture of 50 μg of [3]H-labeled G-30 lipopolysaccharide (100 cpm/μg) and 5 μg of purified enzyme in 0.1 M Tris-HCl (pH 8.5) containing 1 mM EDTA was subjected to gradient centrifugation and fractions were assayed for LPS and ENZ as described in Figures 8 and 9. (b,c) These represent identical experiments except that lipopolysaccharide was replaced by soluble polysaccharide (b) or lipid A (c) derived from 50 μg of [3]H-labeled G-30 lipopolysaccharide as described in Experimental Procedure. The gradient fractions were analyzed for polysaccharide by measurement of radioactivity and for ENZ as described in Figures 8 and 9. The arrows indicate the positions of LPS, ENZ, polysaccharide (P_s), and lipid A when subjected to gradient centrifugation separately. The position of lipid A was estimated by determining the phosphate content of the gradient fractions, but accurate determinations were not possible because of the low content of phosphate and the high concentration of sucrose in the samples. In parts a and b, equilibrium was not reached during the period of centrifugation and the positions of the peaks reflect sedimentation velocities rather than buoyant densities.

amine complex, as shown by formation of a fully active enzyme–lipopolysaccharide–phosphatidylethanolamine complex (Figure 12c). The crude extract did not cause marked changes in the gradient pattern of mixtures of purified transferase and lipopolysacharide in the absence of phospholipid.[3]

Discussion

The role of phospholipid in the galactosyl transferase reaction was clearly related to an effect on the lipopolysaccharide substrate. Activity in the transferase system was seen only with phospholipids which were able to form binary complexes with lipopolysaccharide; phospholipids which did not form binary complexes with lipopolysaccharide (dipalmitoyl-, dihexanoyl-, and hydrogenated *A. agilis* phosphatidylethanolamine) were inactive. The central role of the lipopolysaccharide–phosphatidylethanolamine interaction was most clearly demonstrated by the requirement that the two components be preincubated in order to obtain significant activity, and by the significantly higher yield of the reaction in the presence of phosphatidylethanolamine than in its absence. There was prompt resumption of the reaction when fresh lipopolysaccharide was added after the incubation was permitted to go to completion in the absence of phospholipid (Figure 2), thus eliminating the possibility that phospholipid was merely protecting the enzyme against inactivation.

The effect of phosphatidylethanolamine in increasing the yield of the reaction suggests that the interaction of lipopoly-

[3] Crude enzyme refers to fraction I (sonic extract), as described in the preceding paper (Endo and Rothfield, 1969).

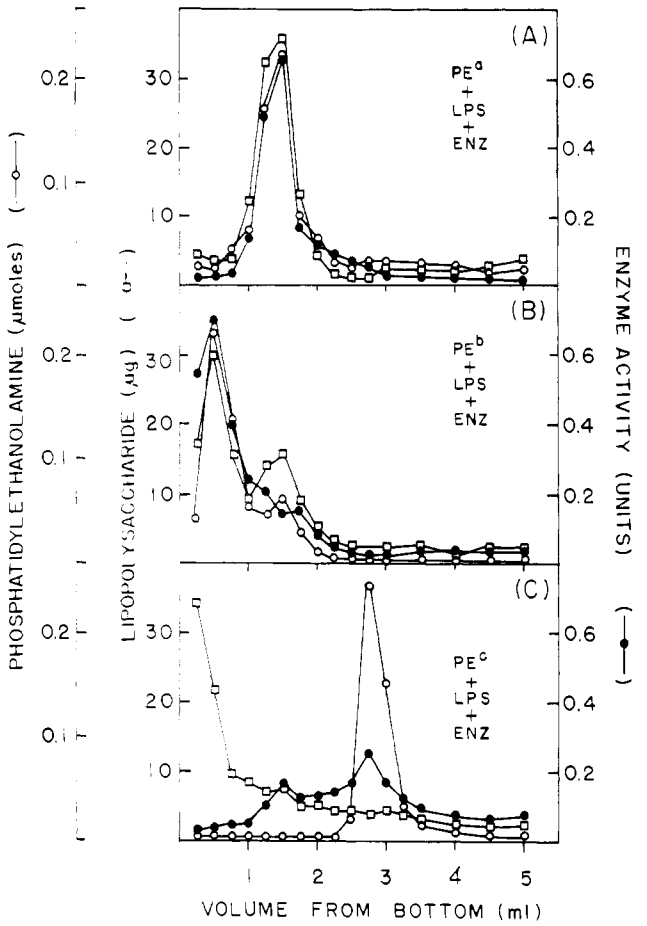

FIGURE 11: Gradient centrifugation of mixtures of lipopolysaccharide, phosphatidylethanolamine, and enzyme. Mixtures of PE and LPS were made as in Figure 8. Approximately 5 μg (70 units) of enzyme was added to the mixture at 0°. Gradient centrifugation and measurement of lipopolysaccharide, phosphatidylethanolamine, and enzyme activity were performed as in Figures 8 and 9. PE[a], PE[b], and PE[c] refer to *A. agilis* phosphatidylethanolamine-^{14}C, didecanoylphosphatidylethanolamine, and hydrogenated *A. agilis* phosphatidylethanolamine-^{14}C, respectively.

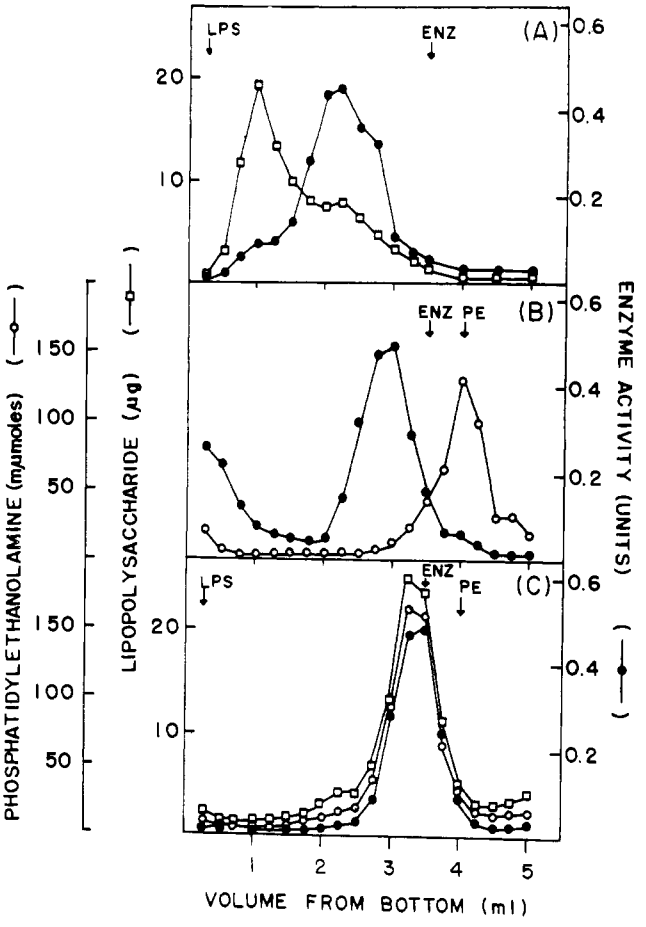

FIGURE 12: Effect of heat-treated crude enzyme on the interaction of enzyme with lipopolysaccharide and phosphatidylethanolamine. Experiments were carried out as in Figures 9, 10, and 11 on the following combinations: (A) purified enzyme and G-30 lipopolysaccharide; (B) purified enzyme and *A. agilis* phosphatidylethanolamine; (C) purified enzyme, G-30 lipopolysaccharide, and *A. agilis* phosphatidylethanolamine. In each case 0.1 ml (0.15 mg of protein) of crude enzyme[3] previously heated at 100° for 10 min was added to each sample immediately prior to centrifugation.

saccharide with phosphatidylethanolamine increased the number of polysaccharide chains available to the enzyme. This could reflect a simple spacing effect in which molecules of lipopolysaccharide are separated from each other by interpolation of phospholipid molecules in a common leaflet structure or could be due to conformational changes of the polysaccharide chains induced by neighboring molecules of phosphatidylethanolamine.

The only kinetic evidence that the phospholipid might also be stimulating the enzyme directly was the finding that the V_{max} of the reaction was significantly higher in the presence of phosphatidylethanolamine than in its absence.

Stimulation of activity of the transferase system occurred only with phospholipids which were able to form complexes with both enzyme and lipopolysaccharide. Thus, no activity was seen with dipalmitoyl- or hydrogenated *A. agilis* phosphatidylethanolamine, which formed complexes with enzyme but not with lipopolysaccharide. Conversely, didecanoylphosphatidylethanolamine, which formed a complex with lipopolysaccharide but not with enzyme, was also ineffective as an activator of the reaction.

The data are consistent with a speculative model in which the full catalytic activity of the enzyme requires specific binding both to the polysaccharide portion of lipopolysaccharide and to phosphatidylethanolamine.

The presence of a polysaccharide binding site is implied by the fact that the enzyme catalyzes transfer to the polysaccharide portion of the molecule. The presence of a polysaccharide binding site was also indicated by the finding that several nonsubstrate lipopolysaccharides were inhibitors of the reaction, and that this inhibition was not seen with mutant lipopolysaccharides which lacked the polysaccharide portion of the molecule (Endo and Rothfield, 1969). It was further supported by the observation that the polysaccharide portion of rough B lipopolysaccharide was an inhibitor of the reaction while the lipid A portion of the molecule was not (see preceding paper, Table V). In the case of the one lipopolysaccharide for which adequate kinetic data could be obtained the

inhibition appeared to be of the competitive type (see preceding paper, Figure 2).

The model predicts that phosphatidylethanolamine plays two roles. (1) The phospholipid interacts with lipopolysaccharide to form a lipopolysaccharide–phospholipid complex whereby binding sites on the polysaccharide are made accessible to the enzyme. Both didecanoyl- and *A. agilis* phosphatidylethanolamine are presumed to be effective in this role since both compounds formed binary complexes with lipopolysaccharide, and both binary complexes were also capable of binding the enzyme (Figures 8 and 11). The failure of dipalmitoyl- and hydrogenated *A. agilis* phosphatidylethanolamine to be active in the enzyme system is ascribed to their inability to form binary complexes with lipopolysaccharide (Figure 8). (2) Phosphatidylethanolamine provides a second binding site for the enzyme. Didecanoylphosphatidylethanolamine is presumed to be ineffective in this role as indicated by its failure to bind to the transferase enzyme (Figure 9b) in the absence of lipopolysaccharide.

Binding to both the polysaccharide and phospholipid are considered necessary for the full catalytic activity of the enzyme. If the model is correct, the inhibition of the transfer reaction by didecanoylphosphatidylethanolamine can be ascribed to removal of lipopolysaccharide from the reaction by formation of lipopolysaccharide–phosphatidylethanolamine complexes which are inactive because of the inability of the phospholipid portion of the complex to interact effectively with the enzyme. Both the decreased yield of the reaction (Figure 6) and the competitive nature of the kinetics (Figure 7) are consistent with this view.

The evidence in support of the scheme is indirect and the model is presented here as a guide to further experimentation.

References

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The Isolation and Identification of 25-Hydroxyergocalciferol*

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ABSTRACT: A polar metabolite of vitamin D_2 (314 μg) has been isolated in pure form from the blood of four pigs given 500,000 IU of ergocalciferol/day for 26 days. It has been identified as 25-hydroxyergocalciferol by means of ultraviolet spectra, gas–liquid partition chromatography, nuclear magnetic resonance spectra, mass spectra, and mass spectra of its trimethylsilyl ether derivative. It is about 1.5 times more active than vitamin D_3 or D_2 in curing rickets in rats.

Lund and DeLuca (1966) described for the first time a polar metabolite of vitamin D_3 which appeared to be at least as biologically active as the parent vitamin in curing rickets in rats. This metabolite was described in man (DeLuca *et al.*, 1967; Avioli *et al.*, 1967), chicks (Drescher *et al.*, 1969), and hogs (Blunt *et al.*, 1968a), proved to possess all the biological activities of the parent vitamin, and acted more rapidly (Lund and DeLuca, 1966; Morii *et al.*, 1967). This metabolite was isolated in pure form and identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). Evidence has been obtained that it represents the metabolically active form of the vitamin (Blunt *et al.*, 1968c; Trummel *et al.*, 1969).

Recently it was shown that the peak IV metabolite is also formed from vitamin D_2 in both rats and chicks (Drescher *et al.*, 1969). This metabolite has now been demonstrated in hogs, isolated in pure form, and identified as 25-hydroxyergocalciferol. It is the purpose of this communication to report these findings.

Methods and Results

General Procedures. All radioactive determinations were carried out by means of a Packard Tri-Carb Model 3003 liquid scintillation counter equipped with an automatic external standardization system. Samples to be counted were evaporated to dryness with a stream of air, dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene] per l. of toluene), and counted.

Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Samples in this case were dissolved in diethyl ether.

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