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**LA THÈSE A ÉTÉ
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Lysophosphoglyceride interconversions in
Escherichia coli and other
Gram-negative bacteria

by

Isabel Fuentes-Inostroza

A thesis

Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of Master of Science

Department of Biochemistry
University of Ottawa

January, 1979

© I. Fuentes-Inostroza, Ottawa, Canada, 1979.

Avec amour,

A mon époux Julio
et à mes enfants
Rodrigo, Gonzalo
Patricia et Cecilia.

ACKNOWLEDGEMENTS

My gratitude is expressed to Professor Pierre Proulx whose knowledge and research experience have been very helpful.

My thanks are extended to the technical assistance of Francine Landry.

This study was supported by the Medical Research Council of Canada.

S U M M A R Y

Escherichia coli particulate fraction were found to catalyze the conversion of lysophosphatidylethanolamine to lysophosphatidylglycerol. The product of the reaction was identified by Thin layer chromatography with different solvent systems and by mild alkaline hydrolysis followed by paper chromatography of the water-soluble product. Lysophosphatidylglycerol formation from $[^{32}\text{P}]$ -lysophosphatidylethanolamine was stimulated by Ca^{++} at low concentrations and inhibited by other divalent cations. The pH optimum for this conversion is 6.8-8.0. The conversion of a mixture of sn-glycero-3 $[^{32}\text{P}]$ -phosphorylethanolamine and 1- $[^{14}\text{C}]$ acyl-sn-glycero-3-phosphorylethanolamine occurred without a change in the $^{14}\text{C}/^{32}\text{P}$ ratio, indicating a direct transfer of the lysophosphatidyl group to a suitable acceptor. Since this conversion was stimulated by DL- α -glycero-phosphate and since sn-glycero-3-phosphate (U) $[^{14}\text{C}]$ readily incorporated into labeled lysophosphatidylglycerol in the presence of added lysophosphatidylethanolamine, the true lysophosphatidyl acceptor is very likely, sn-glycero-3-phosphate. This conclusion was further substantiated by the fact that glycerol $[^{14}\text{C}]$ incorporation into lysophosphatidylglycerol was stimulated by ATP.

In accordance with the evidence presented, we postulate that the mechanism for the formation of lysophosphatidylglycerol involves an exchange reaction between the ethanolamine moiety of lysophosphatidylethanolamine and glycerophosphate. Lysophosphatidylethanolamine addition stimulated the incorporation of ethanolamine- $[^{14}\text{C}]$ but not that of serine- $[^{14}\text{C}]$ into lysophosphatide. No exchange reaction was found when diacylphosphoglycerides were used as substrates. The exchange reaction involving lysophosphatidylethanolamine and glycerophosphate was detected in other Gram-negative bacteria such as Proteus vulgaris and Pseudomonas aeruginosa and was absent in the Gram-positive bacteria such as Bacillus cereus, Bacillus subtilis and Staphylococcus aureus. The significance of these results is discussed in terms of other known phosphoglyceride pathways in Escherichia coli and of homeostatic mechanisms within the membrane.

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I N T R O D U C T I O N

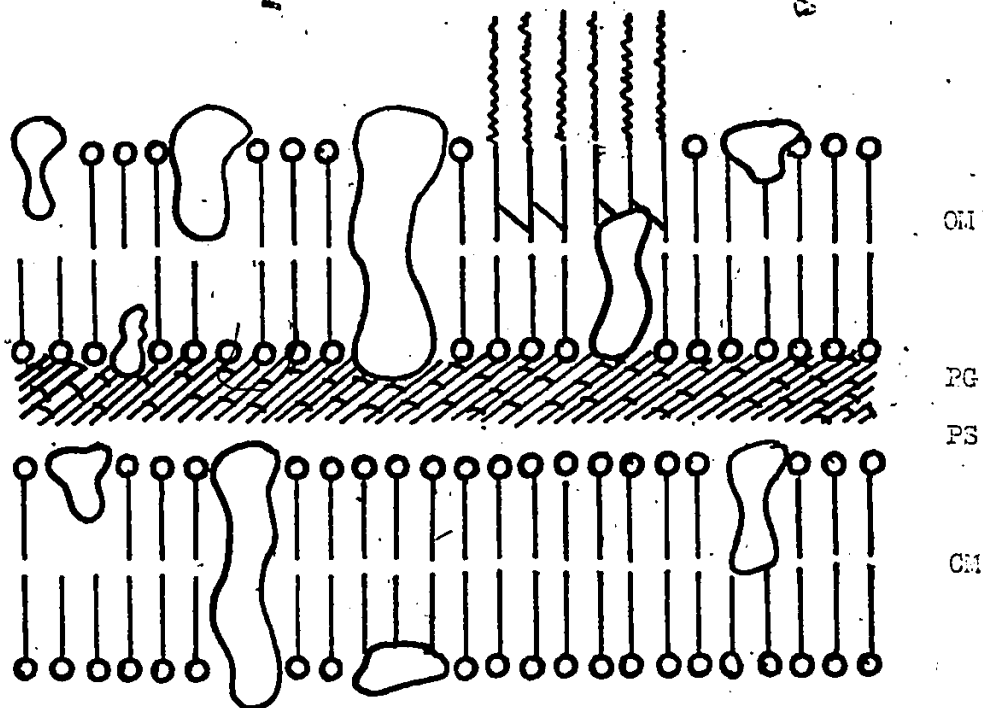
I. General composition of bacterial membranes.

Membranes serve to separate aqueous compartments with different solute composition and they are the structural base to which certain enzymes, various receptors and transport systems are bound.

Bacteria in general have at their surface, a cell membrane and a cell wall; in Gram-positive bacteria these two identities can be distinguished easily by electron microscopy (1); the envelope of Gram-negative bacteria, like that of Escherichia coli, appears to be more complex: it consists of an outer membrane, an inner membrane and an interlayer of peptidoglycan bridging the two membranes as illustrated in Figure 1 (2).

In both Gram-positive and Gram-negative cells, the structure of the walls consists of parallel peptidoglycan polymers cross-linked by peptide bond involvement. The muropeptide is the basic recurring unit in the peptidoglycan and consists of a disaccharide of N-acetyl-D glucosamine and N-acetyl muramic acid in β (1 \rightarrow 4) linkage (see figure 2a).

The peptidoglycan layer imparts stability and shape to the cell and assists the molecular sieving processes of the membranes. There is a minute space between this muropeptide



PHOSPHOLIPID PROTEIN UNITS OF
TEICHOIC POLYSACCHARIDE
JOINED BY PYRO-
PHOSPHATE BRIDGES

OM= outer membrane
PG= peptidoglycan

PS= periplasmic space
CM= cytoplasmic membrane

FIGURE 1

Schematic cross-sectional view of
Escherichia coli membrane.

N-acetylglucosamine

N-acetylmuramic acid

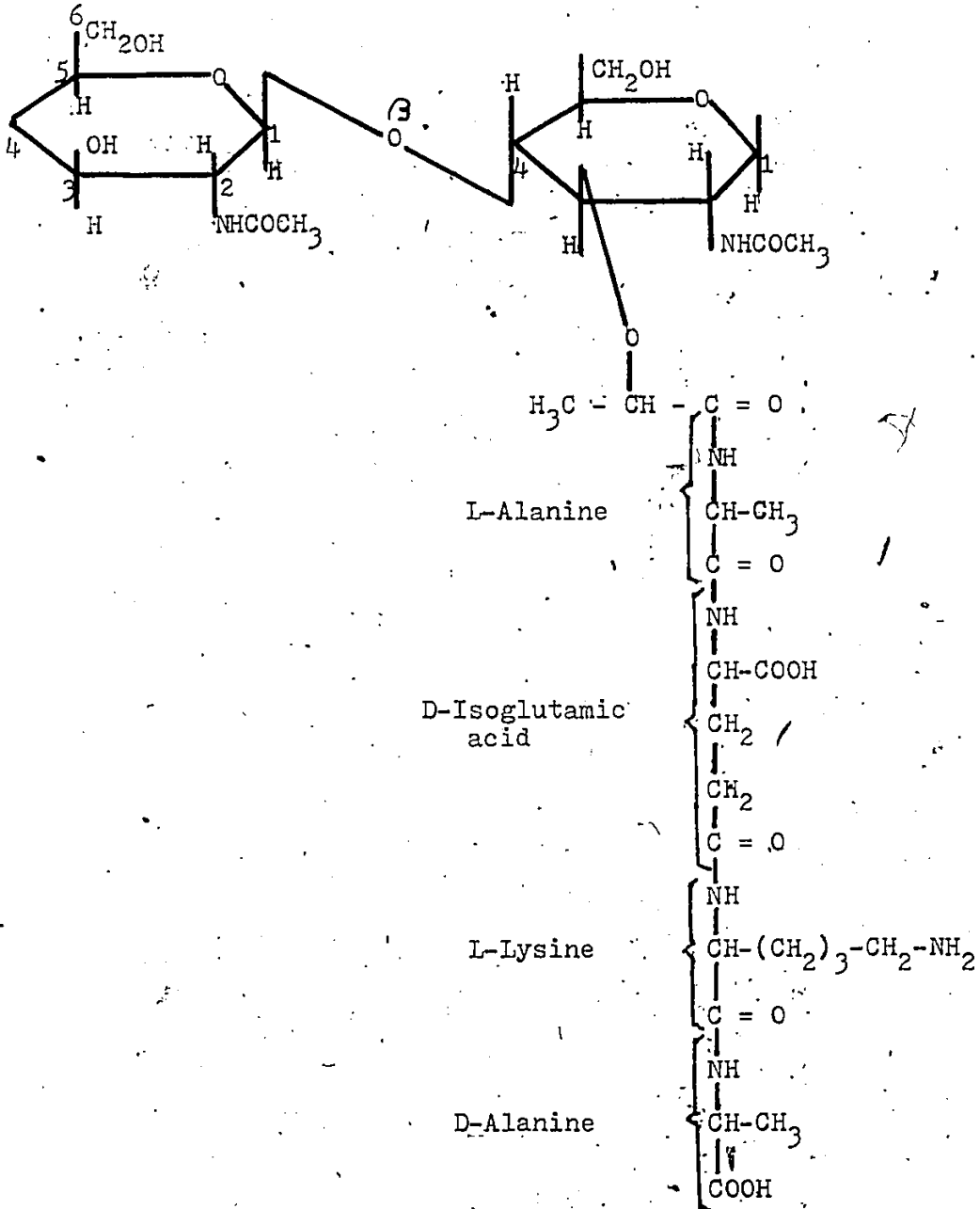


FIGURE 2a

Recurring muropeptide of bacterial cell walls.

layer and the inner membrane, the periplasmic space which contains numerous hydrolytic enzymes and transport binding proteins (3).

The membranes consist of about equal proportions of proteins and lipids, the outer membrane containing about 60% of the total lipid. The protein composition of each membrane is quite different (4). The protein composition of the inner membrane is much more complex than that of the outer membrane. It comprises many of the biosynthetic enzymes including those for lipid synthesis as well as the permeant and electron transport carriers and the ATPase involved in oxidative phosphorylation. It appears that all the components of the Gram-negative envelope are made on the inner membrane or in the cytoplasm. Appropriate components are subsequently translocated across the inner membrane to the mureopeptide layer or to the outer membrane where they are finally assembled (5,6).

The outer membrane of Gram-negative bacteria typically contains lipopolysaccharide, lipoprotein, protein and lipid (7,8). The lipopolysaccharide consists of three entities covalently linked (Figure 2b). There is the O antigen region which is the outermost portion of the polymer and is constituted by a repeating series of oligosaccharides, quite variable in composition depending on the strain examined. It confers typical antigenic properties to the strain. The core portion has an outer core region which is fairly variable in carbohydrate composition and an inner core quite stable in its complement of constituent carbohydrate units.

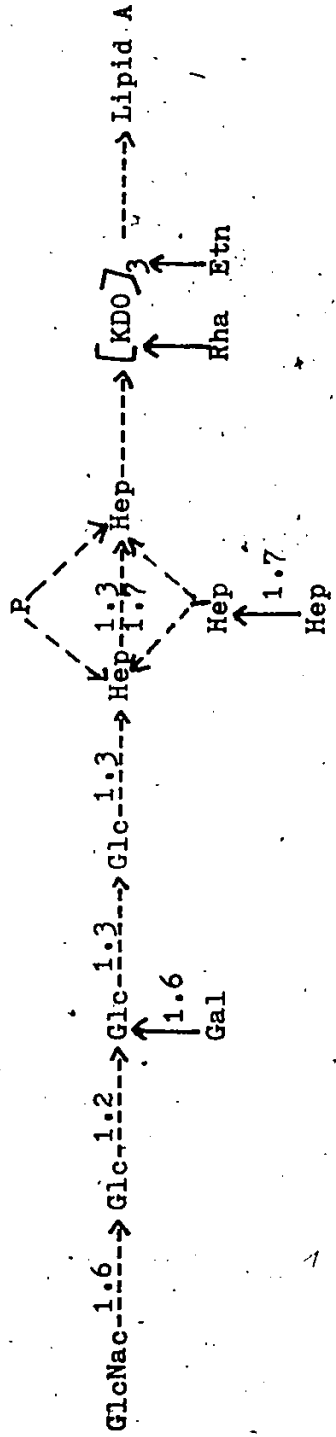


FIGURE 2b

Probable structure of lipopolysaccharide
of Escherichia coli.

This inner core region contains pyrophosphoryl ethanolamine and 2-keto-deoxy-octonate as typical constituents. The innermost region consists of lipid A, a complex structure containing glucosamine, fatty acids and esterified phosphate(9).

I.1. Structural models of the coliform membranes.

Cellular membranes differ widely in their morphology and functions, but there are some general principles that can be applied to all membranes, whether they are from prokaryotic or eukaryotic cells. All membranes contain polar lipids arranged as bilayer and this bilayer architecture accommodates various amounts of protein (10).

Many models have been proposed to explain how proteins and lipids are arranged in the biomembranes. These have pictured the lipid bilayer "sandwiched" between two layers of protein as proposed for many years by Davson and Danielli (11), Stein (12) and Robertson (13) but the fluid mosaic model postulated by Singer (14) illustrated in Figure 3 is the most widely accepted. The model simply proposes that the lipids are arranged as a bilayer, the acyl chains being in a liquid crystalline state. Some proteins are intercalated within the bilayer and occasionally may even span the thickness of the membrane and expose groups on both faces. These are the integral proteins. Each membrane has a complement of peripheral proteins which lie on the surface of the membrane, and interact with integral proteins or with

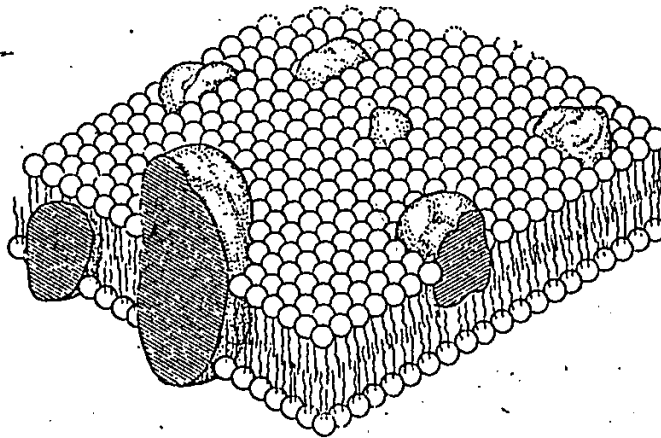


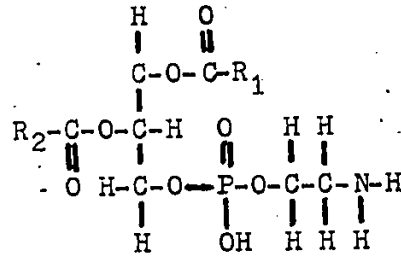
FIGURE 3

Fluid mosaic model of membrane
structure (from S.J.Singer
and G.L.Nicolson Science
175:720 (1972)).

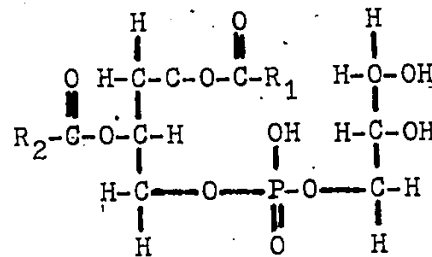
lipid. The proteins are free to rotate and to diffuse in the plane of the membrane because of the semi-fluid nature of the bilayer. In the case of Escherichia coli it is not unlikely that the model applies for both types of membranes, the outer and inner membrane (see Figure 1). The outer membrane is very asymmetrical in structure, the exterior face being much more heavily laden with protein than the inner face (15).

II. The lipids of Escherichia coli.

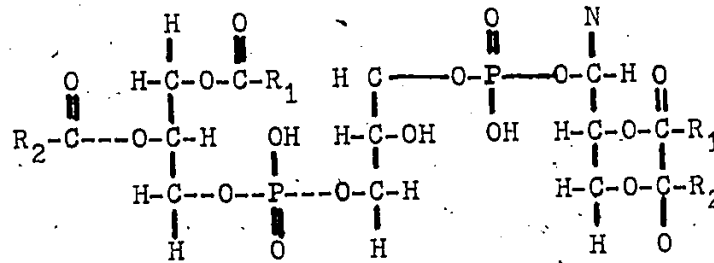
The lipids of Escherichia coli, comprising about one-tenth of the dry weight of the cell, are found in the cell envelope and consist mainly of phospholipids (16) that have amphipathic properties allowing them to form bilayers in aqueous media (14). The chief classes of phospholipids found in Escherichia coli are derivatives of sn-3-phosphatidic acid and consist of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) the structures of which are shown in Fig. 4. Besides these major lipids, trace amounts of free fatty acids are found together with diglycerides, isoprenyl phosphates and minor classes of phosphoglycerides such as phosphatidic acid, phosphatidylserine, phosphatidylglycerophosphate and acyl phosphatidylglycerol which serve as transient anabolic intermediates for the most part.



sn 3-phosphatidylethanolamine



sn 3-phosphatidyl-1'-sn-glycerol



1',3'-di(sn 3-phosphatidyl)-sn glycerol

FIGURE 4

Structure of the major phosphoglycerides
of Escherichia coli.

III. Fatty acid composition of Escherichia coli lipids.

The pool of free fatty acids in Escherichia coli is very small (17), most of the fatty acids being covalently bound to the phosphoglyceride fraction. The saturated fatty acids in Escherichia coli consist mainly of palmitic and myristic acids and traces of stearic and lauric acids. Palmitic acid comprises about the half of the total fatty acid of the cell and is found esterified to acyl ester position 1 of the phosphoglycerides.

The unsaturated fatty acids found in Escherichia coli have been identified as palmitoleic and cis-vaccenic acids (18). Palmitoleic and cis-vaccenic acids are found esterified mainly to position 2 of the phosphoglycerides.

The cyclopropane fatty acids of Escherichia coli are formed by methylation of phospholipid-bound unsaturated fatty acids and they consist of cis-9,10-methylene hexadecanoic acid and cis-11,12-methylene octadecanoic acid. Because of their peculiar mechanism of formation, these fatty acids are also found in the 2 position of phosphoglycerides (19).

The fatty acid composition is greatly influenced by such factors as the culture medium composition, the temperature of growth and the age of the culture (19-24). The fluidity of the membrane which is known to affect a number of enzyme and transport activities in Escherichia coli can be maintained relatively constant by varying the fatty acid composition.

Growth at higher temperatures results in a higher saturated fatty acid content whereas exposure to lower temperatures causes an increase in unsaturated phosphoglyceride species (20,21).

III.1. Biosynthesis of fatty acids.

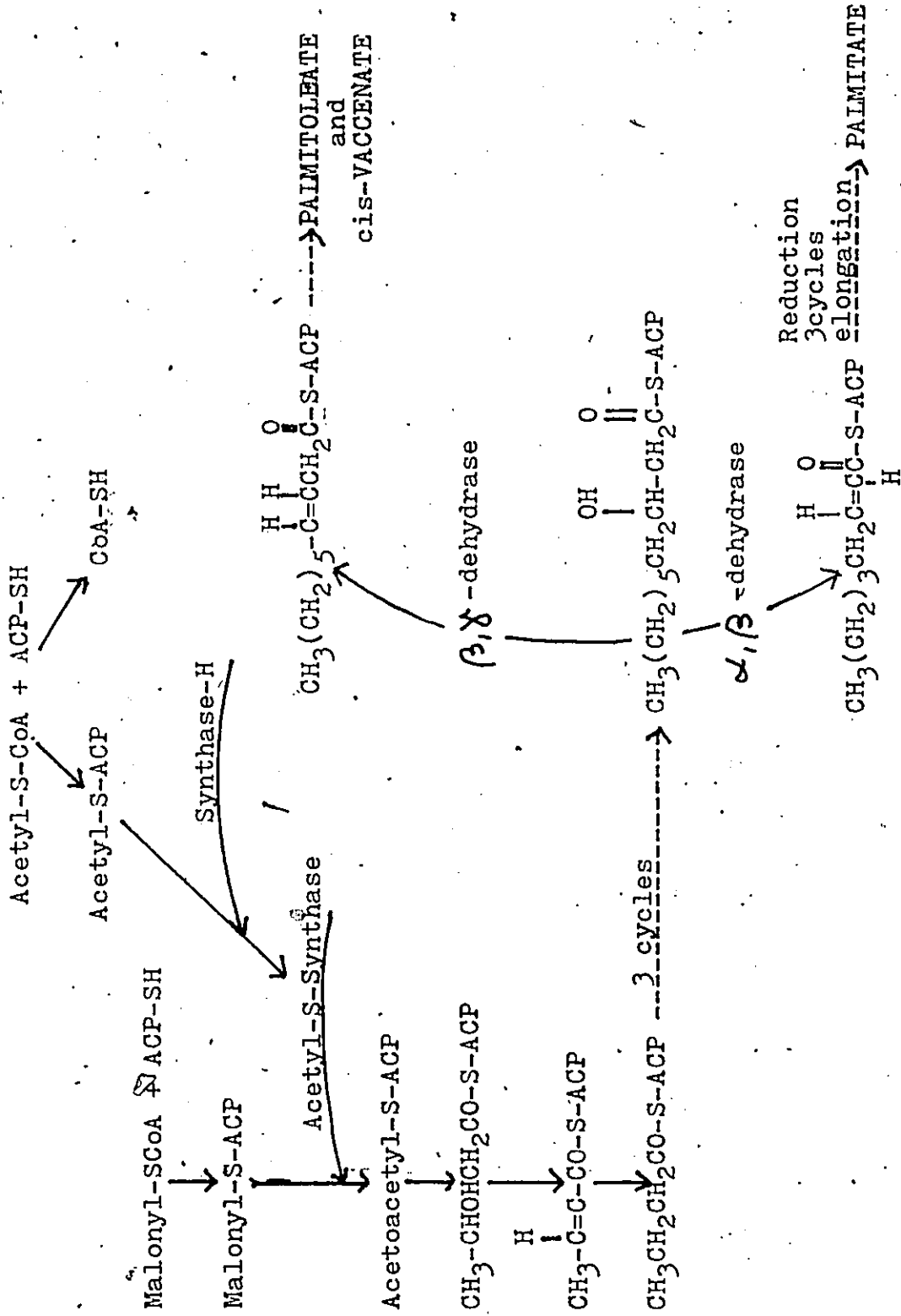
The pathway for the synthesis of fatty acids is summarized in scheme I. The steps involved have been extensively reviewed (25). Interesting is the fact that a specific hydroxy decanoyl acyl carrier protein dehydrase catalyzes the formation of the double bond in the C₁₀ intermediate. This dehydrase is situated at a branch point leading either to the fully saturated palmitic acid or to unsaturated palmitoleic and cis-vaccenic acids.

IV. Phosphoglycerides of Escherichia coli.

In Escherichia coli the phosphoglycerides serve a structural role mainly. They are also associated specifically with a number of proteins which they assist in their biological functions.

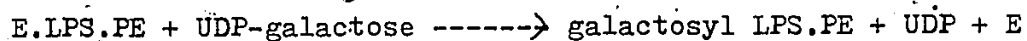
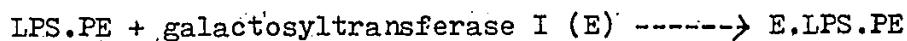
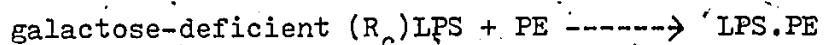
IV.1. Phosphatidylethanolamine.

Phosphatidylethanolamine is the major phosphoglyceride of Escherichia coli, corresponding to 70 - 80% of the total lipids of the cell (16,22,26). The main function of phosphatidylethanolamine is structural but it may serve a role in the bio-



SCHEME I. Pathways for the synthesis of fatty acids.

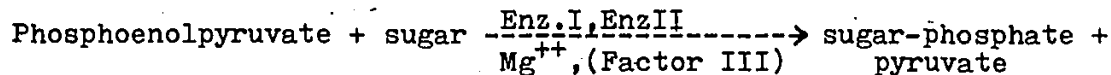
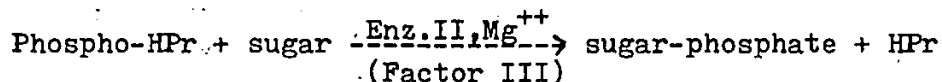
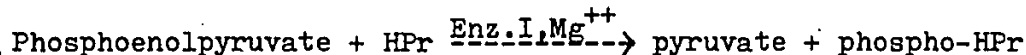
synthesis of lipopolysaccharide of Escherichia coli acting in the transfer of sugar residues from a nucleoside sugar precursor to the non-reducing end of the polysaccharide chain (27). The enzymes involved in the biosynthesis of the R-core of polysaccharides (glucosyltransferase I, galactosyltransferase I) require phosphatidylethanolamine. A binary complex is formed by interaction of phosphatidylethanolamine with lipopolysaccharide in the presence of the transferase and Mg^{++} . This binary complex forms a ternary complex with the enzyme which transfers the glycosyl residue. This function of phosphatidylethanolamine can be summarized in the following reactions (28):



Although phosphatidylethanolamine serves a structural function and may assist in certain catalytic functions, it seems that mutants lacking the ability to form phosphatidylethanolamine and substituting phosphatidylglycerol for this lipid, can survive and grow normally (29).

IV.2. Phosphatidylglycerol

Phosphatidylglycerol comprises 5-15% of the total phospholipids in Escherichia coli (30). Phosphatidylglycerol is implicated in the transport of sugars (31,32), being a component of Enzyme II which is involved in the Phosphoenolpyruvate-phosphotransferase system.



HPr is a low molecular weight, heat-stable protein and like Enzyme I, is a soluble protein (33,34). Enzyme II appears to form an integral part of the membrane, and is responsible for the specificity of the system towards the sugar (32). Enzyme II has three components: two proteins (II-A and II-B) and phosphatidylglycerol (35,36). In some species, not in Escherichia coli, a factor III is required and replaces protein II-A.

Phosphatidylglycerol is precursor in the synthesis of

cardiolipin (37,38) and of several classes of glycerophosphate-containing oligosaccharides (39,40).

Recently (41) it has been reported that phosphatidylglycerol stimulates the activity of the acyl-CoA:sn-glycerol-3-phosphate O-acyl transferase. This enzyme is responsible for the acylation of the glycerophosphate, the first step in the synthesis of phospholipids. Phosphatidylglycerol is also involved in the activation of respiratory carriers (42).

The structure of phosphatidylglycerol is illustrated in Figure 4:

IV.3. Cardiolipin.

Cardiolipin accounts for about 5-15% of the phospholipids of Escherichia coli (30). The role of cardiolipin seems to be structural; but there is evidence indicating its activation of respiratory carriers and may thus play an important role in respiration (42).

The levels of cardiolipin increase substantially in aging cells or in energy depressed cells (43). The significance of this phenomenon is not yet understood.

IV.4. Other phosphoglycerides.

The occurrence of minor phospholipids such as phosphatidylserine, lysophosphatidylethanolamine, phosphatidic acid, phosphatidylglycerophosphate (44), CDP-diglyceride (30,45) and

phosphoisoprenoids (46), have been reported. The main function of these minor phospholipids seems to be that they serve as intermediates in the biosynthesis of the major phospholipids.

Phosphoisoprenoids serve as lipid carriers in the biosynthesis of peptidoglycans and polysaccharide material in bacteria and animals (47).

Acyl phosphatidylglycerol has been reported to be a minor lipid of Escherichia coli and accumulates when particulate fractions of Escherichia coli are incubated with phosphatidylglycerol, Ca^{++} and ether (48). The function of this lipid as well as the precise mechanism for its formation are still under investigation.

V. Biosynthesis of phospholipids in bacteria.

V.1. General principles.

The lipid composition of Escherichia coli is the same if glucose, glycerol, or acetate are used as carbon source in the growth medium, but some factors can affect it. During the transition of Escherichia coli from exponential growth to the stationary growth phase an increase in cardiolipin level and a corresponding decrease in phosphatidylglycerol content occur (23,30,45). A similar result is found when Escherichia coli cells are exposed to an energy-free medium: the level of cardiolipin increases at the expense of phosphatidylglycerol (49). The conversion of

phosphatidylglycerol to cardiolipin is also found when cells are grown in the presence of factors affecting oxidative phosphorylation or energy metabolism (30,50).

The biosynthesis of phospholipids in Escherichia coli has been studied specially by Kennedy and coworkers (51-55). The reactions involved in this biosynthesis are described in Scheme II.

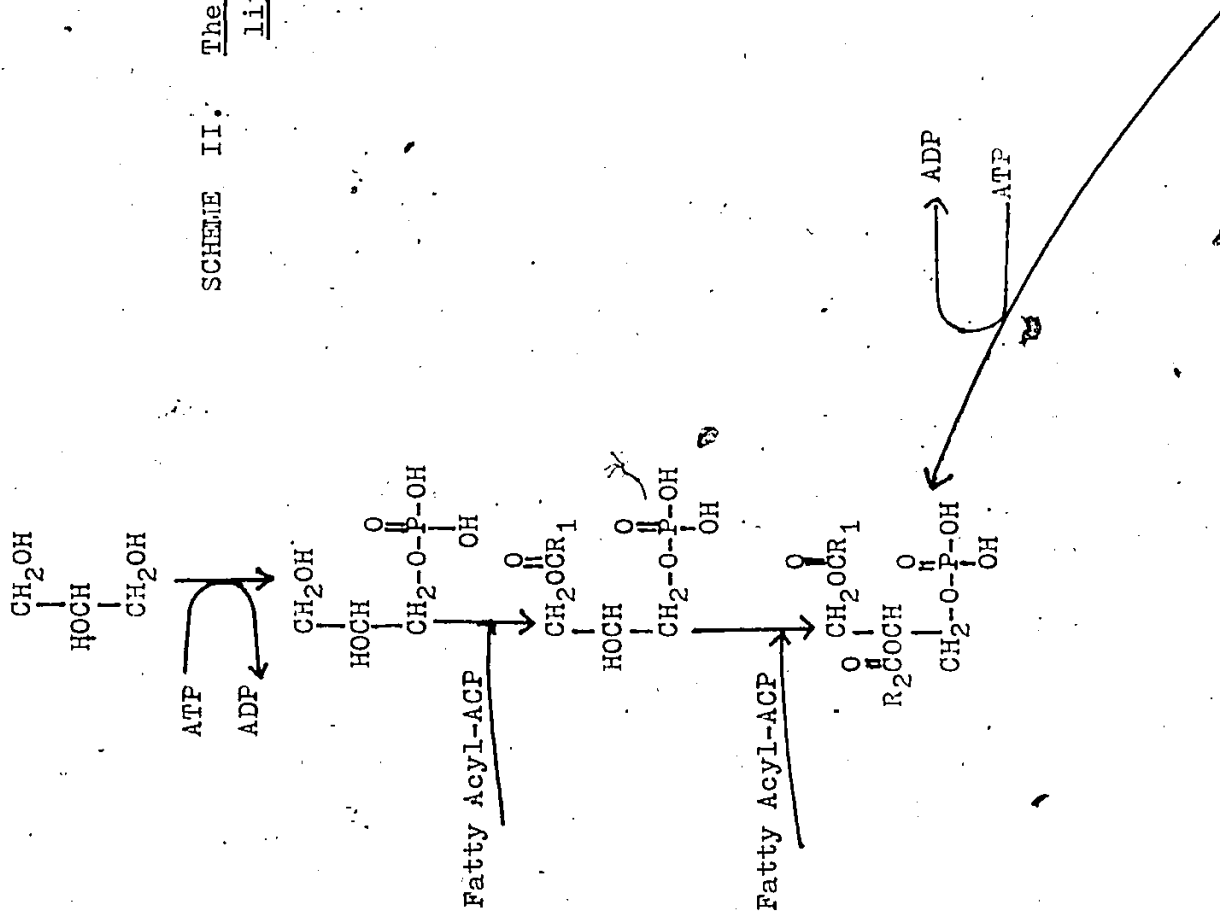
V.2. Synthesis of phosphatidic acid.

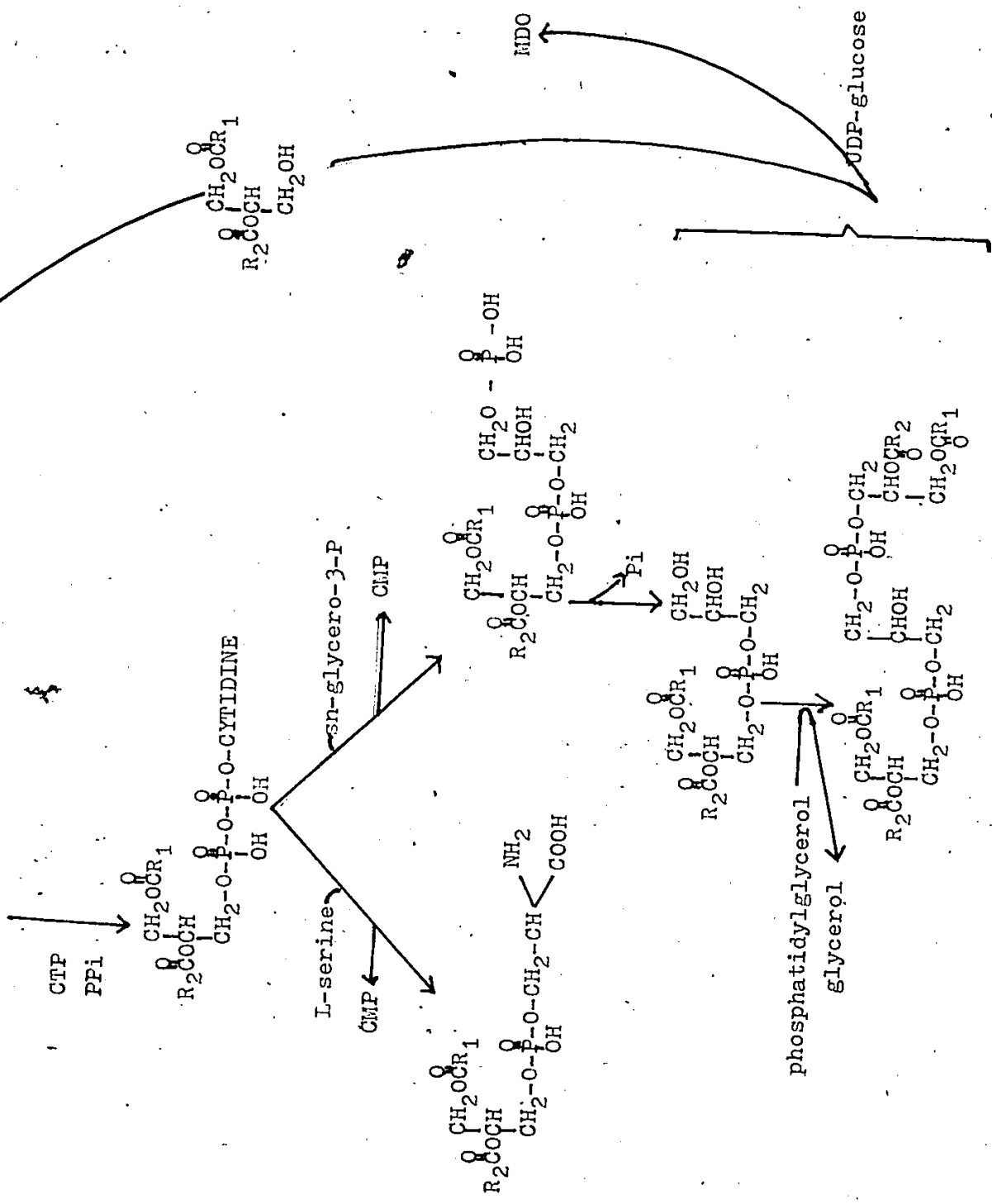
V.2.1. Acylation of sn-glycerol-3-phosphate.

Phosphatidic acid is formed by acylation of sn-glycerol-3-phosphate. The enzyme involved in this process is the glycerophosphate acyl transferase which was partially purified by Ishinaga, et al. (56) and by Snider and Kennedy (57). These investigators found that the enzyme required phosphatidylglycerol (56) or a mixture of phosphatidylethanolamine and phosphatidylglycerol (57) for activity.

Acylation of sn-glycerol phosphate is thought to be the step at which the fatty acid composition of the phospholipids is determined. In Escherichia coli the saturated fatty acids are found mainly in position 1 and the unsaturated fatty acids mainly in position 2 of the glycerophosphate moiety and this specific distribution has been attributed to the specificity of the glycerophosphate acyltransferase. It was reported (19,58) that when glycerol-3-phosphate was acylated with palmitoyl CoA,

SCHEME II: The biosynthesis of the phospholipids of Escherichia coli.





the product was 1-acyl glycerol-3-phosphate, and when an unsaturated fatty acyl CoA was used for the acylation, 2-acyl glycerol-3-phosphate was obtained; the same result was found when the acylation was carried out with a mixture of saturated and unsaturated acyl CoA (59). However, other workers (56, 57, 60, 61) found that unsaturated and saturated CoA are incorporated as 1-acyl glycerophosphate.

Formation of position-specific phospholipids in the absence of a rigidly specific acylation process can be explained on the basis of different rates of acylation with saturated and unsaturated acyl CoA. It has been postulated that the acylation with saturated acyl CoA proceeds faster than the acylation with unsaturated acyl CoA (59, 60, 62, 63) and the incorporation of a second acyl group to the 1-acyl glycerophosphate is faster with an unsaturated acyl CoA than with a saturated acyl CoA.

The acylation of glycerol-3-phosphate, *in vitro*, may be carried out by either acyl-CoA or acyl-ACP, with different specificities (58, 62). *In vivo* it is not clear whether CoA or ACP is the acyl donor in phospholipid synthesis. Mutants of Escherichia coli lacking acyl-CoA synthetase could incorporate endogenous fatty acids into phospholipids but were unable to incorporate exogenous fatty acids (64). On this basis it was thought that exogenous fatty acids are incorporated by way of CoA and endogenous fatty acids by ACP, but it was later found (65) that acyl-CoA synthetase mutants were unable to incorporate

exogenous fatty acids. Ray and Cronan (66) demonstrated, in Escherichia coli, the presence of an enzyme responsible of the conversion of fatty acid to acyl-ACP. The role of this enzyme is unknown but it seems to be involved in the incorporation of free fatty acid into phospholipids. No intracellular acyl-CoA has been detected in Escherichia coli cells (65), probably due to the presence, in these cells, of two thioesterases very active on acyl-CoA substrates (67,68). On the other hand indirect evidence has been reported (69) for the presence of intracellular acyl-ACP in Escherichia coli, suggesting that in vivo, the acylation is carried out by acyl-ACP rather than with acyl-CoA.

V.2.2. Phosphorylation of diglyceride.

Inner membrane of Escherichia coli also contains a kinase which converts sn-1,2-diglyceride to phosphatidic acid. Diglyceride kinase has been extracted from membranes and purified 600 fold (70). It catalyzes the phosphorylation of diglyceride, monoglyceride, ceramide and other such analogues (70,71).

The role of this enzyme is uncertain since it appears that acylation of sn-glycero-3-phosphate is the major de novo pathway for phosphatidic acid synthesis in vivo (72). Diglyceride is a very minor pool of lipid in Escherichia coli and does not turn over rapidly (52,73) as compared to phosphatidic acid and CDP-diglyceride generally accepted as being a more important phosphoglyceride precursor (52,74).

Mutants lacking diglyceride kinase accumulate sizeable amounts of diglyceride (up to 10 % of the total lipids) which must mean that the enzyme does play some role in the channeling of neutral lipid to phosphoglycerides. The diglyceride accumulating in these mutants may arise from phosphatidylglycerol or from cardiolipin as suggested by Raetz (73).

Kennedy and coworkers reported that most of the turnover of polyglycerophosphatides could be accounted for by the donation of an *sn*-glycero 1'-phosphate mainly from phosphatidylglycerol or cardiolipin to a family of oligosaccharides containing glucose, succinate, *sn*-glycero 1'-phosphate and pyrophosphorylethanolamine. The by-product of this reaction would be diglyceride (75,76).

V.3. Synthesis of CDP-diglyceride.

The accepted pathway for the synthesis of CDP-diglyceride in Escherichia coli is the activation of phosphatidic acid by CTP (77-79). The enzyme involved in this reaction, phosphatidic acid cytidyltransferase, was found to be located in the inner membrane of Escherichia coli (80,81).

CDP-diglyceride and deoxy CDP (dCDP) diglyceride have been implicated as donors of the phosphatidyl units in the synthesis of phosphatidylglycerol and phosphatidylserine (51,54). Raetz and Kennedy (74) showed that the liponucleotide fraction of

Escherichia coli is a mixture of CDP-diglyceride and dCDP-diglyceride but their levels in vivo are very low. This can be explained on the basis of an active, specific hydrolase in Escherichia coli which catalyzes the hydrolysis of CDP-diglyceride. Also the conversion of these liponucleotides to phosphoglycerides is extremely rapid. This probably means that the enzymatic step for CDP-diglyceride formation is rate-limiting.

CDP-diglyceride hydrolase, partially purified by Raetz et al. (82,83) is present in the membrane fraction and catalyzes the hydrolysis of CDP-diglyceride to phosphatidic acid and CMP. This enzyme is not very active with dCDP-diglyceride and one could therefore suggest that it functions in the regulation of the CDP-diglyceride to dCDP-diglyceride ratio in vivo.

CDP-diglyceride synthetase requires phosphatidic acid with unsaturated fatty acids, being inactive with dipalmitoyl phosphatidic acid. In Escherichia coli 60-80% of the liponucleotide fraction is CDP-diglyceride whereas the remainder is accounted for by the deoxy analogue.

V.4. Synthesis of phosphatidylglycerol.

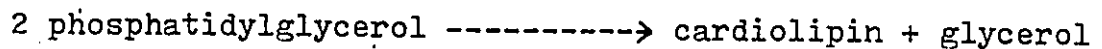
Phosphatidylglycerol is formed according to the following reaction (51) :

synthetase. The phosphatidylglycerophosphate synthetase is very active only with CDP-diglyceride or its deoxy analogue as substrates; other liponucleotides being ineffective as substrates. The dCDP-diglyceride is almost twice as effective as CDP-diglyceride at high concentrations (67).

The phosphatidylglycerophosphate phosphatase is also a particulate enzyme stimulated by Triton X-100. It requires Mg^{++} and is specific for phosphatidylglycerophosphate. Its very marked activity in vivo would explain why phosphatidylglycerophosphate does not accumulate in this organism.

V.5. Synthesis of cardiolipin.

Cardiolipin is synthesized according to the following reaction (37,38,49,85,86):

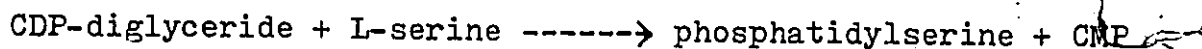


Stanacev et al. (53) had proposed that cardiolipin is synthesized in Escherichia coli by a reaction similar to that involved in the synthesis of cardiolipin in mammalian mitochondria utilizing phosphatidylglycerol and CDP-diglyceride, but evidence for this reaction in Escherichia coli has not yet been provided. The transphosphatidyltion reaction appears to be the only mechanism involved; although, CDP-diglyceride does stimulate the

conversion of phosphatidylglycerol to cardiolipin some three-fold (37).

V.6. Synthesis of phosphatidylserine.

Phosphatidylserine is synthesized according to the following equation:



This reaction is catalyzed by CDP-diglyceride L-serine phosphatidyltransferase (PS synthetase), the optimal activity of which depends on a surface active agent (octanol or Triton X-100) and a high ionic strength. The purification of this enzyme bound to the ribosomes in extracts of Escherichia coli has been reported (87).

Homogenous phosphatidylserine synthetase not only transfers the phosphatidyl group from CDP-diglyceride to L-serine but also exchanges free CMP with the CMP moiety of CDP-diglyceride and L-serine with phosphatidylserine (87,88). The reversal of phosphatidylserine synthesis occurs in the presence of phosphatidylserine and CMP but the equilibrium is in favor of the phosphodiester lipid. The enzyme also hydrolyzes CDP-diglyceride and phosphatidylserine at a slow rate to form phosphatidic acid (87,88). CDP-diglyceride and dCDP-diglyceride are the best substrates for phosphatidylserine synthetase although

UDP-diglyceride and ADP-diglyceride are also active as substrates (87,88).

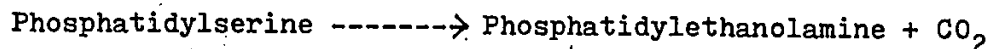
The enzyme also catalyzes the formation of phosphatidyl-glycerophosphate when serine is replaced by glycerophosphate but at a slow rate. Glycerol is a poor phosphatidyl acceptor if used in molar quantities (87).

Phosphatidylserine is found only in trace amounts in Escherichia coli likely because it is rapidly converted to phosphatidylethanolamine. Evidence that phosphatidylserine synthetase is the main enzyme involved in phosphatidylethanolamine synthesis in vivo comes from genetic studies. Mutants defective in this enzyme have a decreased phosphatidylethanolamine content and a higher proportion of polyglycerophosphatides. However this genetic evidence does not preclude the possibility of alternative pathways of phosphatidylethanolamine synthesis in vivo. In fact some mutants have been isolated which neither synthesize phosphatidylethanolamine nor accumulate phosphatidylserine. These mutants seem to have a normal phosphatidylserine synthetase and their mutations are on the genetic map at a position distinct from phosphatidylserine synthetase mutations (89-91).

V.7. Synthesis of phosphatidylethanolamine.

The synthesis of phosphatidylethanolamine involves the decarboxylation of phosphatidylserine as illustrated in the

following reaction:



The reaction is catalyzed by phosphatidylserine decarboxylase which has been isolated from Escherichia coli and purified (92).

The reaction illustrated above has been demonstrated to occur by Kanfer and Kennedy (51) who found that in the presence of hydroxylamine, an inhibitor of the phosphatidylserine decarboxylase, Escherichia coli cells accumulated [^{14}C] from [^{14}C]-serine into phosphatidylserine. In the absence of hydroxylamine, [^{14}C]-phosphatidylethanolamine was found.

V.8. Lysophosphoglycerides.

Lysophosphoglycerides have been reported to occur in trace amounts in Escherichia coli (23,93,94). The lysophosphoglycerides are obtained as intermediates in phospholipid metabolism. Lysophosphatidic acid is obtained as intermediate in phosphatidic acid synthesis (95,96). Lysophosphoglycerides can also be obtained by hydrolysis of diacylphospholipids with phospholipase A, present in different strains of Escherichia coli (97,98).

V.9. Regulation of phospholipid synthesis.

It is not definitely known which mechanisms regulate phospholipid synthesis. Glaser et al. (99) indicated that the synthesis of lipids is coupled to the synthesis of other molecules. When temperature-sensitive glycerophosphate-acyltransferase mutants were subjected to the restrictive temperature not only phospholipid formation stopped but DNA, RNA and protein synthesis also stopped. On the other hand, when cells were starved for a required amino acid not only did protein and RNA synthesis cease but that of phospholipids also (100).

There is normally a tight coupling of phospholipid and fatty acid synthesis in bacteria (17,30). Starving of glycerol auxotrophs results in an inhibition of both fatty acid and phospholipid synthesis (101). The mechanism of inhibition is not clearly known, although the blocking of an early step in fatty acid synthesis has been suggested (101). There could well occur a mechanism for regulating phosphatidic acid and CDP-diglyceride formation. However mutants which are overproducers of acyltransferases do not synthesize greater amounts of phospholipids. It seems that the synthesis is not directly controlled by enzyme levels.

Nunn and Cronan (102) found that high concentrations of guanosine 5'-diphosphate-3'-diphosphate (ppGpp) inhibited

the phospholipids synthesis and it was found that one of the inhibited enzymes was the glycerophosphate acyltransferase (102 - 104). The other was the phosphatidylglycerophosphate synthetase. In vitro studies showed that inhibition was found only when acyl-CoA is the acylating substrate and no inhibition was found when the acylating substrate is acyl-ACP (58,103). Since the inhibition by ppGpp is irreversible and reversible inhibition has been observed in vivo (102,104) it is not certain whether ppGpp is a true regulator substance. Cronan has suggested that it probably is not (29).

The ratio of polar headgroups in Escherichia coli membranes is rigidly controlled. The phosphatidylethanolamine-polyglycerophosphatide ratio is always approximately 3:1. Even when the decarboxylation of phosphatidylserine is blocked, the sum of phosphatidylserine plus phosphatidylethanolamine content divided by the amount of polyglycerophosphatide is the same as for normally functioning cells (105). There is no definite knowledge concerning the regulatory mechanism involved in keeping the polar headgroups ratio relatively constant. The involvement of simple molecules such as ppGpp is at the moment very uncertain.

Separate pools of phosphatidic acid have been reported to exist in membrane preparations but other studies have indicated that there is a single pool of phosphatidic acid and liponucleotide that can be used by both branches of

the pathway (106). It appears that both phosphatidyltransferases involved utilize CDP-diglyceride and its deoxy analogue equally well so that the preferred use of one liponucleotide by a particular branch is not a factor in the regulation.

The relative levels of the phosphatidyltransferases appears not to be a factor since overproducers of phosphatidylserine synthetase do not synthesize a larger proportion of phosphatidylethanolamine. The availability of serine and L-glycerophosphate could be a factor which could be tested by growing auxotrophs on limiting concentrations of L-serine and sn-glycerol-3-phosphate. This point needs clarification.

The lipid composition in the membrane and the net charge that results could be a factor influencing the activity of the phosphatidyltransferases. This possibility has not yet been tested with the purified enzymes and liposomes of different compositions.

Another factor that could possibly intervene is the exchange of bases that would occur between lipid classes. The conversion of one class to another could be influenced by the availability of water-soluble substrates such as serine, ethanolamine, and/or sn-glycerol-3-phosphate. The possible existence of base exchange reactions in Escherichia coli has not been investigated systematically (79,87) and such will be the object of the present study.

VI. The catabolism of glycerophosphatides.

Phospholipids can be hydrolyzed by more or less specific phospholipases present in Escherichia coli.

VI.1. Phospholipase A and lysophospholipase.

Phospholipase A hydrolyzes an acyl ester bond in phosphoglycerides. Two types of phospholipases A have been described, phospholipase A₁ and phospholipase A₂ which remove acyl groups from the 1- and from the 2-position respectively. Phospholipase A₂ is the more specific and best characterized of phospholipases A found in nature and is present in snake venom of *Crotalus adamanteus* (107,108), of *Naja naja* (109,110), in bee venom (111) and in mammalian tissues (112).

In Escherichia coli several types of phospholipase A have been reported: a detergent-resistant phospholipase A (97) which is bound to the outer membrane (81), a detergent sensitive phospholipase A (97) specific for phosphatidylglycerol and present in the cytosol, an alkaline phospholipase A₁ purified by Scandella and Kornberg (113) first reported by Fung and Proulx (98), an acid phospholipase A₁ and an alkaline phospholipase A₂. It seems that several of these enzyme activities is accountable by a single lipolytic enzyme which purified, displayed phospholipase A₁, phospholipase A₂, lysophospholipase A₁,

and A_2 activities as well as some lipase activity (114,115).

Several lysophospholipase activities have been reported. Doi and Nojima (116) purified to near-homogeneity a lysophospholipase which hydrolyzes 1-acyl phosphatidylethanolamine, 2-acyl phosphatidylethanolamine, 1-acyl phosphatidylglycerol but it is inactive with the diacylphosphatides. Albright et al. (117) reported a lysophospholipase A_1 activity in the cell wall, probably associated with the phospholipase A_1 , a lysophospholipase A_1 in the membrane and cytosol and a lysophospholipase A_2 in the inner membrane.

In addition to these phospholipases, Escherichia coli contains a phosphodiesterase which cleaves glycerophosphoryl-ethanolamine to form sn-glycero-3-phosphate and ethanolamine (117).

VI.2. Phospholipase C.

Phospholipase C hydrolyzes the bond between phosphoric acid and glycerol. Phospholipase C was first discovered in Clostridium perfringis (welchii) by M. Macfarlane (118). Phospholipase C has been purified from Bacillus cereus (119), Pseudomonas fluorescens (120) and some workers have detected phospholipase C activity in certain strains of Escherichia coli (121,122) but this enzyme was not found in most other bacteria studied (123).

VI.3. Phospholipase D.

Phospholipase D removes the polar head of the phospholipids to yield phosphatidic acid. Phospholipase D has been isolated from cabbage leaves (124) and other plant tissues (125). A cardiolipin-specific phosphodiesterase has been found in Escherichia coli and other Gram-negative bacteria, catalyzing a reaction similar to that of phospholipase D (50,126,127). The enzyme, catalyzing the hydrolysis of cardiolipin to yield phosphatidylglycerol and phosphatidic acid, required Mg^{++} for activity, was stimulated by ATP and was inhibited by detergents and EDTA.

VII. The role of phospholipases in Escherichia coli.

The role of most of the lipolytic activities of Escherichia coli remains unknown. Mutants which lack the phospholipase A of outer membrane display no defects in growth (118, 128,129). Several studies have indicated that only the polyglycerophosphatide turns over at an appreciable rate in this organism (16,22,26) and it seems that this turnover is accountable partly by the presence of cardiolipin synthase and phosphodiesterase activities and partly by their conversion to oligosaccharides by enzymes present in the periplasmic space (40). Phospholipase A does not participate in this turnover in normal cells but in

cells with a defective envelope, these phosphoglycerides and phosphatidylethanolamine to an even larger extent are hydrolyzed to yield fatty acids.

It is likely that the phospholipase A activity functions to degrade exogenous phosphoglycerides into simpler molecules which can serve as fuel or as precursors to typical Escherichia coli lipids. The lipase activity of phospholipase A (16) may serve a similar function with respect to exogenous glycerides.

VIII. Sub-cellular localization of biosynthesizing and degradative enzymes.

In Escherichia coli the enzymes involved in the biosynthesis of phosphatidic acid, CDP-diglyceride, phosphatidylglycerol, and cardiolipin are localized in the cell envelope (37,39,52,62,130) and more precisely in the inner membrane of the cell envelope (plasma membrane) (80,81).

Concerning the phosphatidylserine synthetase and phosphatidylserine decarboxylase there is strong evidence showing that phosphatidylserine decarboxylase is located in the plasma membrane (80,81,84,92). The phosphatidylserine synthetase has been reported to be located in the membrane-free supernatant associated with the ribosomes (82,87,92,131). However Cronan (29) has suggested that the true location of this enzyme is on the inner membrane in a loosely bound form.

Association with ribosomes is probably an artifact of the isolation procedure.

The degradative enzymes are present mainly in the outer membrane of Escherichia coli. Bell et al. (81) indicate that phospholipase A, has higher specificity in the outer membrane; Albright et al. (117) succeeded in separating the "cell wall", the inner membrane and the cytosol and they found phospholipase and lysophospholipase A₁ activities in the "cell wall" fraction and a lysophospholipase A₂ activity in the inner membrane.

In spite of the fact that most of the enzymes involved in the phospholipid synthesis are present in the inner membrane of Escherichia coli and that most of the degradative enzymes are located in the outer membrane, it has been found that the phospholipid composition in the inner membrane is similar to that of the outer membrane (80). This implies a translocation of phospholipids from inner to outer membrane of the Escherichia coli cells and possibly a higher turnover rate in the outer membrane.

IX. Base exchange reactions.

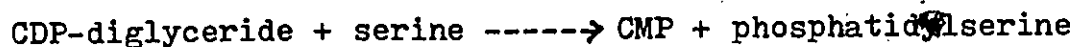
Base exchange reactions have been well characterized in animal tissues.

Working with rat liver homogenate, Hubscher (132) and Borckenhagen (133) found that L-serine was incorporated into lipids in a reaction stimulated by Ca⁺⁺ and not requiring

nucleotide intermediate; this incorporation apparently involved exchange of free serine with the base of endogenous phospholipids. The lipid formed was identified as phosphatidylethanolamine. They also showed incorporation of ethanolamine into phosphatidylethanolamine in the presence of Ca^{++} .

These exchange reactions have been reported to occur also in brain tissue (134-137). The base exchange enzyme system present in animal tissue is tightly bound to the subcellular membranes. It has been postulated (138-140) that this kind of reaction is the result of the transphosphatidylase activity of phospholipase D, but the presence of phospholipase D in animal tissue has not been clearly demonstrated.

In the case of bacteria, exchange reactions have also been studied by some workers. Thomas et al. (79) found that in isolated Escherichia coli membrane vesicles, the serine moiety of phosphatidylserine exchanges with exogenous serine, but it is not clear from the study of Thomas et al. whether it is a direct exchange or a reversal of the following reaction:



However, Larson et al. (87) reported that the phosphatidylserine synthetase purified from Escherichia coli extracts, catalyzed exchange reactions between CMP and CDP-diglyceride and between serine and phosphatidylserine.

It has also been reported (141) that in T. pyriformis,

L-serine is incorporated into phosphatidylserine via exchange reaction with phosphatidylethanolamine.

In our laboratory preliminary evidence was obtained which indicated that lysophosphoglycerides can undergo specific exchange reactions. Experiments to be described in this thesis further characterize these reactions and thus contribute to a more complete knowledge of the metabolism of phosphoglycerides in Escherichia coli.

AIMS OF RESEARCH

1. To characterize in Escherichia coli an base exchange reaction involving lysophosphatidylethanolamine.
2. To determine the pH, cation and substrate requirements of the enzyme(s) catalyzing this type of reaction.
3. To study the occurrence of this enzyme in various bacteria.
4. To investigate the occurrence of other possible base exchange reactions in Escherichia coli.

MATERIALS AND METHODS

I. MATERIALS.

The various bacteria used throughout, Escherichia coli B (ATCC 11303), Proteus vulgaris (ATCC 13315), Pseudomonas aeruginosa (ATCC 10145), Staphylococcus aureus (ATCC 12600), Bacillus subtilis (ATCC 6051) and Bacillus cereus (ATCC 14579) were obtained from the American Type Culture Collection.

ATP (disodium salt) and snake venom from Crotalus adamanteus were purchased from Sigma Chemical Co.

Standard lipids, phosphatidic acid, phosphatidyl-glycerol, phosphatidylethanolamine and cardiolipin were purchased from Serdary Research (London, Canada) or from Sigma Chemicals Co.

[³²P]-orthophosphoric acid, serine-(U)-[¹⁴C], ethanolamine-(U)-[¹⁴C], glycerophosphate-(U)-1,3 [14C] and glycerol-(U)-1,3 [14C] were bought from New England Nuclear Corp.

Reagent grade chloroform, redistilled before use, and other common solvents and reagents purchased from Fisher Scientific Co. mainly, were of the highest grade possible.

II. GENERAL METHODS.

II.1. Preparation of Escherichia coli particulate fraction.

Stock cultures of Escherichia coli B were maintained on nutrient agar slants. Low-form Erlenmeyer flasks containing the culture medium, were inoculated with 5 ml broth culture prepared from stock Escherichia coli slants. The standard culture medium contained in 1 litre : 15 grams of bacto-peptone, 1 gram of yeast extract, 5 grams of sodium chloride and 200 grams of glucose.

The cells were cultured to the late exponential phase (7 hours) and were aerated throughout by gentle agitation of the flasks.

The cells were harvested by centrifugation at 5000 x g for 10 minutes and the sediment was suspended in 25 ml of buffer. This suspension was sonicated for 4 periods of 1 minute with a Biosonik II Ultrasonicator; the unbroken cells were removed by centrifugation at 3000 x g for 10 minutes. The cell-free homogenate was centrifuged at 40,000 x g for 50 minutes and the particulate fraction was washed by resuspension in buffer and centrifugation. The final sediment was suspended usually, in 10 ml. of buffer to give a protein concentration of 4-7 mg/ml.

II.2. Extraction of lipids.

Lipids were extracted from extracts or from chromatograms by the Bligh and Dyer procedure (142).

II.2.1. Lipids from an assay mixture. The incubation mixture, (1 ml aqueous suspension), was mixed with 1.25 ml of chloroform and 2.5 ml of methanol. This mixture was stirred for 2 minutes at room temperature (R.T.) and 1.25 ml of chloroform and 1.25 ml of water were added. The biphasic system was stirred for 1 minute and separated by centrifugation. The lower chloroform layer containing the lipid was transferred to a flask and evaporated to dryness. Evaporation was carried out in a rotatory evaporator under reduced pressure at 37-40°C.

II.2.2. Elution of lipid components from thin layer chromatograms. Each band containing a lipid was scraped from the plate into a centrifuge tube; 5 ml of chloroform and 10 ml of methanol were added; the mixture was stirred for 10 minutes and then 5 ml of chloroform and 5 ml of water were added; the mixture was stirred another 10 minutes and the two phases that had formed were separated by centrifugation.

When acidic lipids such as lysophosphatidylglycerol were to be extracted, the first aliquot of water was replaced by 0.4N HCl and after the final separation of the phases the acidified water-methanol phase was adjusted to pH 7.5 with 0.1N methanolic NH_3 .

II.3. Analytical procedures.

II.3.1. Thin layer chromatography (TLC).

Phospholipids were separated by several thin layer chromatography systems:

System A: A suspension of 50 grams of silica gel G in 50 ml of water was spread as a layer, 0.5 mm thick, on glass plates. These were activated 1 hour at 110°C before use. The chromatograms were developed with chloroform:methanol:water (65:25:4 v/v/v).

System B: This system was similar to system A except that the solvent mixture used was chloroform:methanol:water (65:35:5 v/v/v).

System C: This system was like system A except that the solvent mixture used was chloroform:methanol:acetic acid (65:25:8 v/v/v).

System D: This system was as stated for system A except that the solvent mixture used was chloroform:methanol:ammonia:water (70:30:4:2 v/v/v/v).

System E: A suspension of 50 grams of silica gel H in 100 ml of 1% sodium bicarbonate was spread as a layer, 0.5 mm thick on glass plates. These were activated for 1 hour at 110°C before use. The chromatograms were developed with chloroform:methanol:acetic acid:water (50:25:7:3 v/v/v/v).

System F: This system was similar to system E except that the developing solvent was chloroform:methanol:acetone:acetic acid:water (50:10:20:10:5 v/v/v/v/v).

II.3.2. Paper chromatography.

Ascending paper chromatography served to identify the water-soluble products. For this purpose, Whatman #1 paper was used and the solvents systems were:

System G: Phenol:water (140:35 w/v).

System H: 1M Ammonium acetate pH 7.5:ethanol(35:65 v/v).

System I: Butanol:acetic acid:water (5:4:1 v/v/v).

II.3.3. Localization of lipids.

Generally lipids were revealed by exposure to iodine vapors which following detection, could be removed by aeration.

The nitrogen-containing lipids were localized by spraying with a solution of Ponceau red. This solution contains equal volumes of 0.01% Ponceau red in 0.01N HCl and 0.2% uranyl nitrate in 0.01N HCl (143).

Aminolipids were revealed by spraying with a 0.5% ninhydrin solution in acetone-butanol (1:1 v/v). The chromatograms were heated 5-10 minutes at 110°C after which time, phospholipids containing free amino groups such as phosphatidylethanolamine were revealed as purple spots.

Lipids containing vicinal hydroxyl groups, such as phosphatidylglycerol, were detected by spraying the plates with

2 % sodium metaperiodate and allowed to react 5-10 minutes; the plates were then placed in a tank containing sulfur dioxide, then sprayed with Schiff's reagent, and placed once again in a sulfur dioxide atmosphere. Purple spots developed in the areas containing lipids with vicinal hydroxyl groups.

Schiff's reagent was prepared by dissolving 1 gram of p-rosanilin in 50 ml. of water. The solution was decolorized with sulfur dioxide and diluted to 1000 ml with water.

Radioactive components were detected by scanning the TLC plates with an Actigraph III radioscanner (Nuclear Chicago Corp.).

II.3.4. Mild alkaline hydrolysis.

Mild alkaline hydrolysis of lipids was performed as described by Marshall and Kates (144). The lipids were dissolved in 0.5 ml of methanol:chloroform (3:2 v/v), and to this solution 0.5 ml of 0.2N methanolic NaOH was added. The mixture was allowed to stand at R.T. for 15 minutes. After this time, 1 ml chloroform: methanol (4:1 v/v) and 0.9 ml. of water were added. The mixture was centrifuged at 600 x g for 1 minute, and the upper methanol-water phase was separated and neutralized or slightly acidified with a few drops of Dowex 50 H⁺ resin in the presence of 1 drop of 1% phenolphthalein as indicator. The resin was removed by centrifugation and the supernatant was made slightly alkaline with 1.5M methanolic NH₃. The solution was then evaporated to dryness or to a small volume.

II.4. Radioisotope counting.

Solutions of lipids in chloroform were counted in a scintillation vial containing 5 grams of 2,5-diphenyloxazole, 0.35% acetic acid (v/v) in a mixture toluene-methanol 10:1 (v/v).

When the lipids to be counted were on the thin layer chromatograms, the band containing the lipid was transferred directly from the plate to the scintillation vial and counted.

For the water-soluble radioactive materials, the scintillation fluid was replaced by Aquasol (Nuclear Chicago Corp.):toluene mixture (1:1 v/v).

Radioactivity on the paper chromatograms was measured by counting each component directly. For this purpose the radioactive bands, revealed by scanning the chromatograms, were cut into strips sufficiently large that they could be stood up in the vial

II.5. Other analytical procedures.

II.5.1. Protein determination.

Protein was determined by the procedure of Lowry et al. (145). Particulate fractions were diluted with water. To 1 ml aliquots of the diluted particulate fraction, 5 ml was added of a solution prepared with 0.5 ml of 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5 ml of 2% Na-or K-tartrate and 50 ml of 2% Na_2CO_3 in 0.1N NaOH. The mixture was allowed to stand at R.T. for 10 minutes and 0.5 ml

of phenol reagent (Folin-Ciocalteu) were added. After 60 minutes at R.T. the optical density (O.D.) was read at 680 μm . A standard curve was prepared at the same time with crystalline bovine albumin.

II.5.2. Phosphorus determination.

Phosphorus was determined according to the method of Bartlett (146). The lipids were evaporated to dryness with a jet of nitrogen and 2 ml of water followed by 0.5 ml of 10N H_2SO_4 were added. The tubes were heated at 160°C for 3 hours, after which time 2 drops of 30% H_2O_2 were added to each tube. The heating was continued for at least $1 \frac{1}{2}$ hour. The tubes were then cooled and the volumes were adjusted to 1 ml with water. 5.2 ml of 0.22% ammonium molybdate and 0.3 ml of Fiske-Subbarow reagent were added and the mixture was heated for 7 minutes in a boiling water bath. Absorption was read at 830 μm . A standard curve was prepared with a stock solution of Na_2HPO_4 .

II.5.3. Hydrolysis of phospholipids with phospholipase A.

The hydrolysis of phospholipids by phospholipase A was performed according to the method of Hildebrand (147). Snake venom (3.5 mg/ml) from *Crotalus adamanteus* or *Crotalus atrox* was dissolved in 0.1M Tris-HCl buffer, pH 7.0, containing 0.01M CaCl_2 . The solution was heated at 60°C for 10 minutes to destroy any lysophospholipase activities and then cooled at 20°C ; the pH was adjusted to 7.2. The phospholipid to be hydrolyzed was

evaporated to dryness and dissolved in 2 ml. of ethyl ether. 30 ul of 0.15N NH_4OH , 15 ul of 0.2M SDS and 30 ul of the Tris-HCl buffer containing the CaCl_2 and snake venom were added. The mixture was incubated at R.T. for 4-6 hours with mechanical shaking. The reaction was stopped by addition of 2 ml of ethanol and the mixture was evaporated to dryness.

II.6. Substrates preparation.

II.6.1. Preparation of $[^{32}\text{P}]$ -labeled phospholipids.

$[^{32}\text{P}]$ -labeled phospholipids were prepared by growing Escherichia coli in 1 litre of medium containing 5 mCi of $[^{32}\text{P}]$ -orthophosphate. The composition of the culture medium was the same as described previously. The 1 litre flask was incubated at 37°C for 17 hours with rotatory shaking. The cells were harvested by centrifugation at $5000 \times g$ for 10 minutes and suspended in distilled water; phospholipids were extracted by the method of Bligh and Dyer. The supernatant was collected in a 1 litre flask, adjusted to pH 7.0, autoclaved and, after addition of glucose, the reconstituted culture medium was reinoculated with a new broth to give an additional yield of labeled cells.

The lipids were separated with TLC systems, C and A, successively and identified by mild alkaline hydrolysis followed by paper chromatography of the water-soluble product in phenol: water (system G). The main labeled lipids isolated in this manner were phosphatidylethanolamine approximately 75% of total lipid radioactivity, phosphatidylglycerol (15-20%) and cardiolipin

(5-10%). These yielded the following mild alkaline hydrolysis products, glycerophosphorylethanolamine (Rf 0.60-0.65), glycerophosphorylglycerol (Rf 0.38-0.44) and di-(glycerophosphoryl)-glycerol (Rf 0.08-0.15) respectively.

II.6.2. Preparation of phosphatidyl [^{14}C]-ethanolamine.

Phosphatidyl [^{14}C]-ethanolamine was isolated from cells of Escherichia coli grown in a medium described by Raetz and Kennedy(148), containing 0.11M glycerol, 3 mM MgSO_4 , 2 mM KCl , 0.5 mM K_3PO_4 , 5 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01 mM FeSO_4 and 1.1×10^8 dpm of serine-(U)-[^{14}C]. Phosphatidylethanolamine was separated and purified by TLC in system A; the identity of the lipid was ascertained by mild alkaline hydrolysis and paper chromatography in phenol:water (system G). Mild alkaline hydrolysis also indicated that approximately 15% of the label was present in the fatty acid chains.

II.6.3. Preparation of lysophosphoglycerides.

Labeled or unlabeled lysophosphatidylethanolamine (LPE) and lysophosphatidylglycerol (LPG) were prepared from the phosphatidylethanolamine and phosphatidylglycerol analogues by hydrolysis with phospholipase A according to the procedure of Hildebrand et al. (147) described previously.

Lysophosphatidylethanolamine and lysophosphatidylglycerol were separated and purified by TLC with system A.

R E S U L T S

I. INTERCONVERSION OF LYSOPHOSPHOGLYCERIDES IN GRAM NEGATIVE BACTERIA.

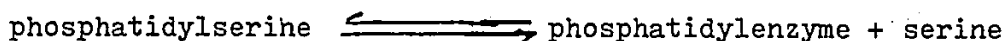
Base exchange reactions have long been known to occur in mammalian tissues.

Thomas et al. (79) indicated that when isolated Escherichia coli membrane vesicles were incubated under conditions favouring phosphatidylserine formation, serine (U)- ^{14}C could be incorporated into membrane-bound phosphatidylserine without the addition of CTP.

This indicated that the serine moiety of phosphatidylserine could exchange with added serine. On the other hand this exchange was stimulated by cytosine monophosphate (CMP) addition. This phosphatidylserine synthetase might have been implicated according the following reaction:



However, it is known that phosphatidylserine synthetase participates in a direct exchange reaction between phosphatidylserine and serine probably according to the following reaction :



Working with a mutant of Escherichia coli lacking glycerokinase (strain 9) Ballesta et al. (149) found that the non-acylated glycerol moiety of phosphatidylglycerol displays a faster rate of turnover than the acylated moiety and they indicated that this is in part, due to the fact that the non-acylated glycerol moiety can exchange with glycerol.

The present study is concerned the possible occurrence of exchange reactions in Escherichia coli. Preliminary studies in our laboratory indicated that when lysophosphatidylethanolamine was incubated with an Escherichia coli particulate fraction, a product hitherto unidentified, binding more firmly to Silica Gel G than lysophosphatidylethanolamine itself, is produced. Identification of this product and characterization of the type of enzymatic reaction involved, together with the cofactor requirements of the reaction have indicated that exchange reactions do occur with lysophosphatidylglycerides in Escherichia coli.

The results to be presented in the following section give a detailed account of these findings.

I.1. (³²P) - lysophosphatidylglycerol formation.

Escherichia coli particulate fractions prepared as stated in the General Methods, were incubated for 30 minutes at 37°C with [³²P]-lysophosphatidylethanolamine in

the presence of Ca^{++} . A product was obtained which, following thin layer chromatography in several systems, displayed an Rf value lower than lysophosphatidylethanolamine.

The unknown product was identified as the intact lipid by thin layer chromatography with the aid of several solvent systems and by mild alkaline hydrolysis followed by paper chromatography of the water-soluble product.

Results summarized in Table I compare the chromatographic properties of this product with those of other phosphoglyceride standards. These results indicate that the unknown lipid is lysophosphatidylglycerol.

Results presented in Table II further show that mild alkaline hydrolysis of the product yielded glycerophosphorylglycerol as could be ascertained from the Rf values obtained with the three paper chromatography systems used.

On this basis, one can propose that at least one enzyme exists which catalyzes base exchange reactions with lysophosphoglycerides and thus converts lysophosphatidylethanolamine to lysophosphatidylglycerol.

TABLE I

Chromatographic properties of the uncharacterized lipid product compared to those of other phosphoglycerides.

Phosphoglyceride	Rf values on different chromatographic systems					
	A	B	C	D	E	F
Uncharacterized lipid	0.21	0.24	0.21	0.40		
Phosphatidic acid	0.00	0.01	0.67	0.07		
Phosphatidylglycerol	0.36	0.40	0.56	0.58		
Phosphatidyl-ethanolamine	0.56	0.73	0.35			
Cardiolipin	0.76	0.79	0.95	0.52		
Lysophosphatidyl-glycerol	0.20	0.26	0.23	0.40		
Lysophosphatidyl-ethanolamine	0.33	0.46				
Lysophosphatidic acid					0.46	0.20

TABLE II

Chromatographic properties of the mild alkaline hydrolysis product of the uncharacterized lipid compared to those of known deacylation products.

	Rf values on different chromatographic systems		
	G	H	I
Deacylated uncharacterized lipid	0.40	0.83	0.29
Glycerophosphoryl-glycerol	0.40	0.81	0.30
Glycerophosphate	0.29	0.27	0.25
Glycerophosphoryl-ethanolamine	0.63	0.22	
Glycerophosphoryl-glycerophosphate	0.15	0.50	0.17

I.2. The effect of protein concentration on the formation of ^{32}P -lysophosphatidylglycerol.

The effect of the protein concentration on the conversion of [^{32}P]-lysophosphatidylethanolamine to [^{32}P]-lysophosphatidylglycerol was studied. Results in Figure 5 indicate that under the incubation conditions used, the reaction was linear up to a concentration of 3 mg/ml and no other major lipid products were formed. A small amount of labeled phosphatidylethanolamine accumulated at higher protein concentrations.

I.3. Effect of pH on the lysophosphatidylglycerol formation.

The effect of pH on the formation of lysophosphatidylglycerol is illustrated in Figure 6. The enzyme has a broad optimum between pH 6.8 and 8.

I.4. Cation requirement for the enzymatic reaction.

The cation requirement for the conversion of lysophosphatidylethanolamine into lysophosphatidylglycerol was also determined. Results summarized in Table III reveal that there is formation of lysophosphatidylglycerol without the

FIGURE 5

The effect of protein concentration on lysophosphatidylglycerol formation. The incubation mixture contained in 1 ml, 0.15 μmol of [^{32}P]-lysophosphatidylethanolamine (22 850 dpm), 5mM CaCl_2 , different amounts of Escherichia coli particulate fraction and 0.1 M Tris buffer pH 7.3 . Incubation was for 30 minutes at 37°C.

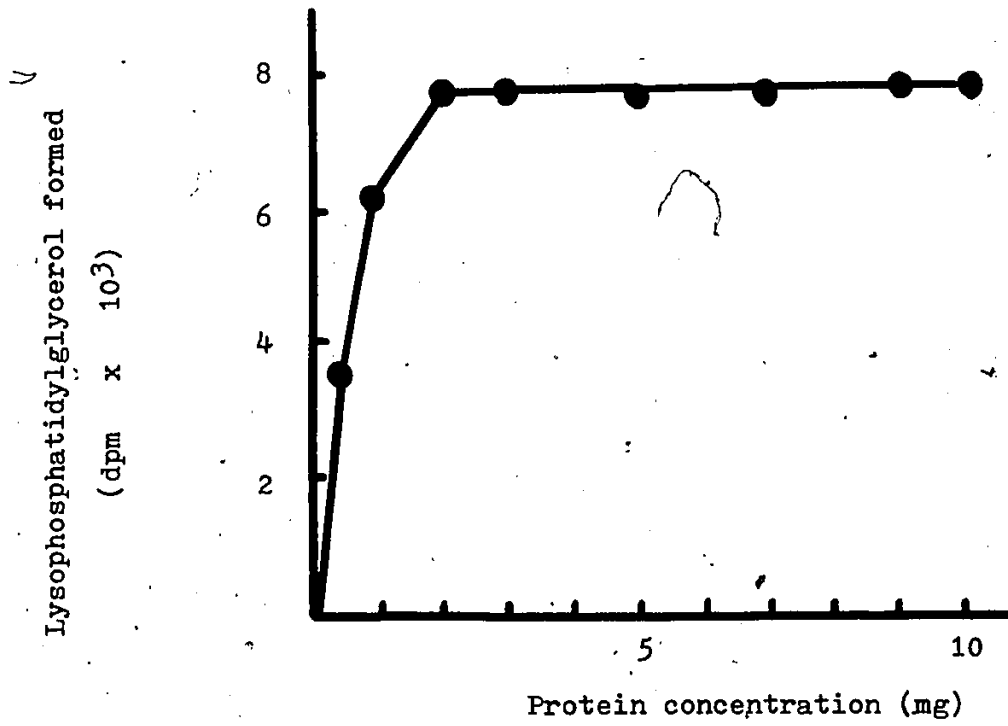


FIGURE 5

FIGURE 6

pH curve for
enzymatic reaction of lysophos-
phatidylglycerol formation. The
assay mixture contained in 1 ml,
1 mg of particulate fraction protein,
0.1 μ mol [32 P]-lysophosphatidyl-
ethanolamine (18 670 dpm), 5 mM CaCl_2 .
In the pH range 4-7, the buffer was
0.1 M acetate and in the pH range 7-9
0.1 M Tris-HCl buffer was used.

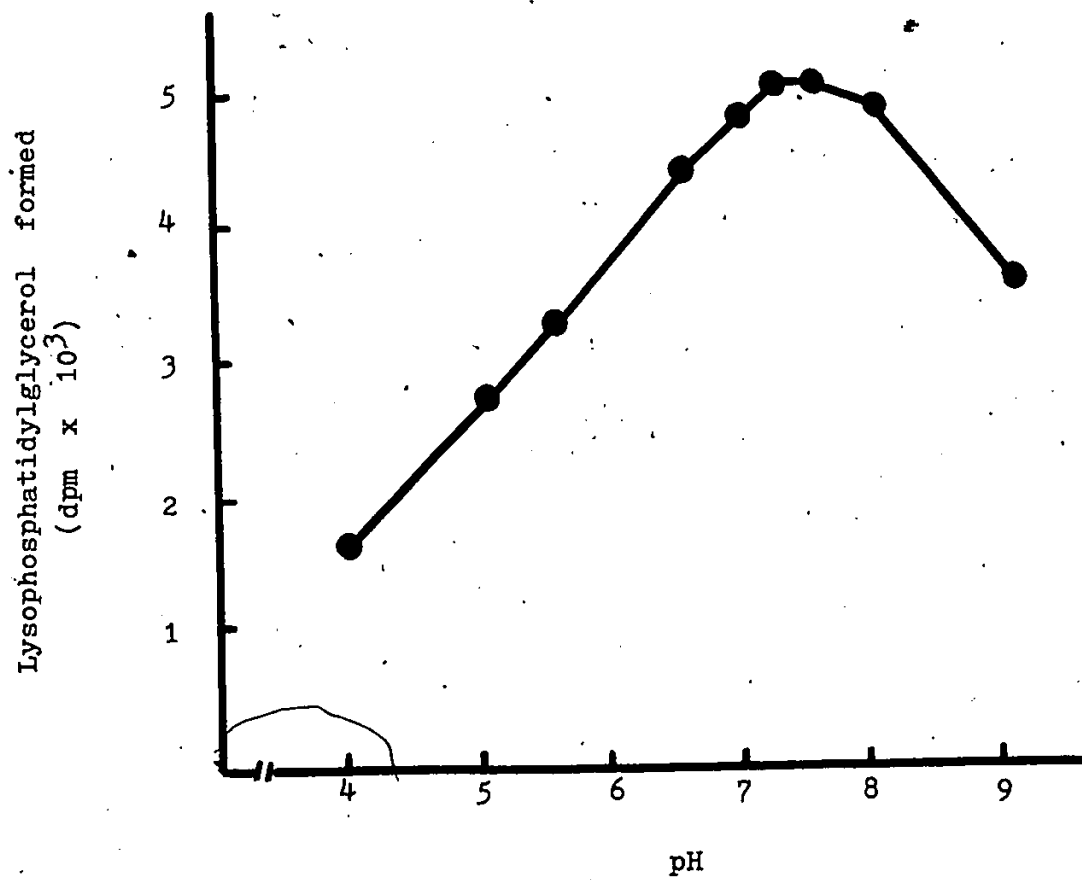


FIGURE 6

TABLE III

The incubation mixture contained, in 1 ml, 0.1 M Tris buffer pH 7.3, 0.9 mg particulate fraction protein, 0.1 μmol [^{32}P] -lysophosphatidylethanolamine and the cations as indicated.

TABLE III

The effect of cations on lysophosphatidylglycerol formation.

Conditions	Counts recovered in lysophosphatidylglycerol
No cation added	4 283 (1 200)*
Ca ⁺⁺ (2.5 mM)	5 630 (2 500)*
(5.0 mM)	5 875 (2 800)*
Mg ⁺⁺ (2.5 mM)	1 748
(5.0 mM)	1 230
EDTA(10 mM)	0
Mn ⁺⁺ (2.5 mM)	0
(5.0 mM)	0
Cu ⁺⁺ (2.5 mM)	0
(5.0 mM)	0
Cd ⁺⁺ (2.5 mM)	0
(5.0 mM)	0

* Results obtained from another similar experiment.

addition of any cation but the lysophosphatidylglycerol formed under these conditions varied with the extent of washing or dilution of the enzyme.

The conversion of lysophosphatidylethanolamine to lysophatidylglycerol was stimulated by Ca^{++} and inhibited by the other divalent cations studied. EDTA inhibited the reaction completely. The optimal concentration of Ca^{++} was found to be 5-8 mM. Marked inhibition of the reaction occurred at concentration above 10 mM (cf. Figure 7).

I.5. Conversion of [^{14}C]- and [^{32}P]-lysophosphatidyl-ethanolamine to lysophosphatidylglycerol.

Doubly-labeled lysophosphatidylethanolamine was incubated with Escherichia coli particulate fraction, under the established conditions of pH and cation, in order to test whether the conversion of lysophosphatidylethanolamine to lysophosphatidylglycerol occurred with a change in the $^{14}\text{C}/^{32}\text{P}$ counts ratio. The results illustrated in Table IV indicate that lysophosphatidylglycerol was formed without a change in this ratio, suggesting that the formation of this lipid product occurred by a transfer of the intact lysophosphatidyl group to a suitable acceptor.

FIGURE 7

Effect of Ca^{++}
concentration on the lyso-
phosphatidylglycerol formation.
The incubation mixture contained,
in 1 ml, 0.1 M Tris buffer pH 7.3,
0.7 mg of particulate fraction
protein, 0.05 umoles [^{32}P]- lyso-
phosphatidylethanolamine and
 CaCl_2 as indicated.

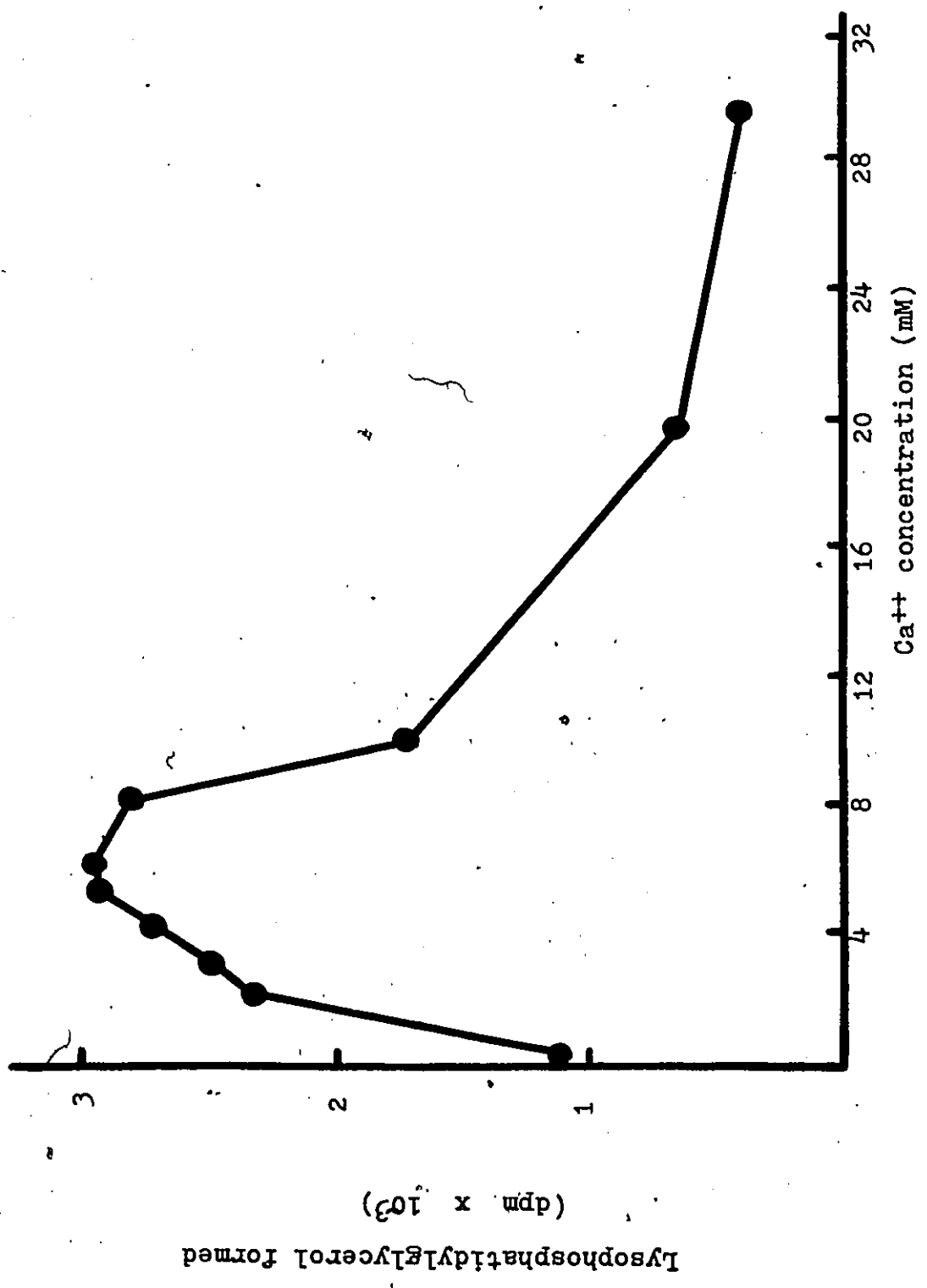


FIGURE 7

TABLE IV

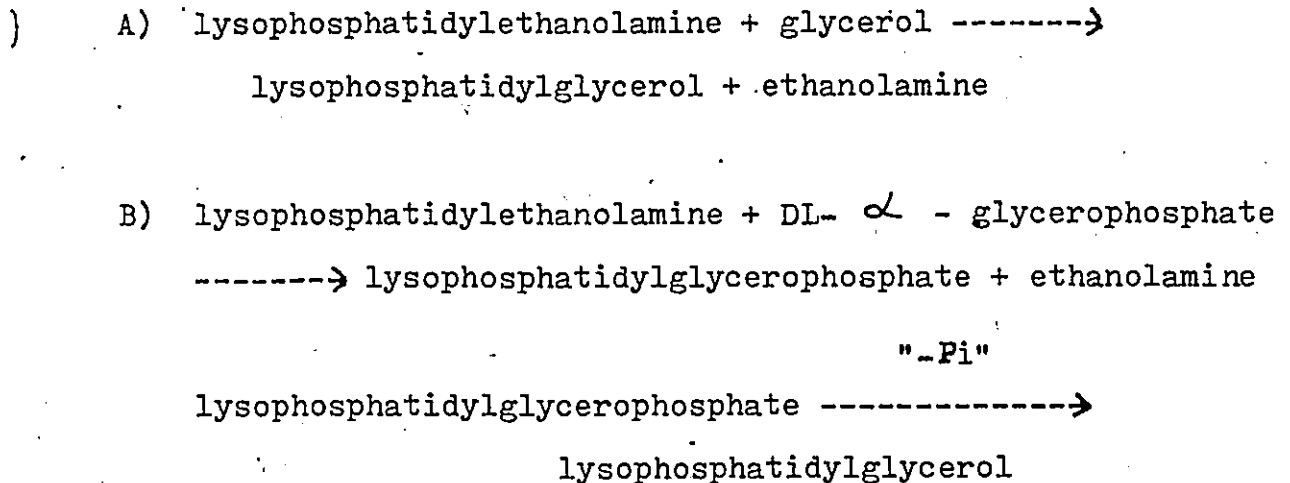
Conversion of ^{14}C and ^{32}P - lysophosphatidylethanol-
amine to lysophosphatidylglycerol.

	^{14}C	^{32}P	$^{14}\text{C}/^{32}\text{P}$ ratio
Lysophosphatidyl- ethanolamine	29 780	18 970	1.57
Lysophosphatidyl- glycerol	8 600	5 690	1.51

The incubation mixture contained in 1 ml, 0.1 M Tris buffer pH 7.3, 5 mM CaCl_2 , 1.1mg of particulate fraction protein, 24 nmoles ^{32}P -acylglycerophosphoryl-ethanolamine (18 970 dpm) mixed with 38 nmoles of 1- ^{14}C acylglycerophosphorylethanolamine (29 780 dpm).

I.6. The effect of glycerol and glycerophosphate on the conversion of lysophosphatidylethanolamine into lysophosphatidylglycerol.

Lysophosphatidylglycerol could be formed by either or both of the reactions illustrated below :



Results illustrated in table V indicate that lysophosphatidylglycerol formation is not markedly stimulated by exogenous glycerol. However it is likely that endogenous pools of this substrate are generated during incubation, via phosphoglyceride breakdown. Addition of ATP to the incubation mixture caused an enhanced stimulation by added glycerol which indicated that glycerophosphate is a better substrate for the reaction. Accordingly, results in Table V reveal a much more significant stimulation by glycerophosphate than by glycerol.

On the basis of these results one can propose that

TABLE V

(A) The incubation mixture contained in 1 ml, 0.1 M Tris buffer pH 7.3, 1.1 mg of particulate fraction protein, 0.15 μmol [^{32}P] lysophosphatidylethanolamine, 5 mM CaCl_2 .

(B) The conditions were the same as those stated for (A) except that glycerol and ATP were added as indicated.

(C) The conditions are the same as those stated for (A) except that DL - α - glycerophosphate was added as indicated.

TABLE V

The effect of glycerol and sn-1(3)-glycerophosphate on
the conversion of lysophosphatidylethanolamine
to lysophosphatidylglycerol.

Conditions	Counts recovered in lysophosphatidylglycerol
(A) Ca ⁺⁺ , no glycerol added	7 610
(B) Glycerol 4%	7 820
Glycerol 6%	8 140
Glycerol 4% + ATP (2.5 mM)	8 510
Glycerol 6% + ATP (2.5 mM)	8 920
(C) DL- α -glycerophosphate (10 mM)	10 560
DL- α -glycerophosphate (15mM)	12 610

mechanism B mainly, is responsible for lysophosphatidyl-glycerol formation. However mechanism A, perhaps catalyzed by another enzyme, cannot be precluded.

I.7. Incorporation of $[^{14}\text{C}]$ -glycerol and $[^{14}\text{C}]$ -glycerophosphate into lysophosphatidylglycerol and the effect of lysophosphatidylethanolamine addition.

Since DL- α -glycerophosphate stimulated the conversion of lysophosphatidylethanolamine to lysophosphatidylglycerol, DL- α -glycerophosphate was pictured as the true substrate for this reaction. This possibility was further tested by incubating Escherichia coli particulate fraction with sn-glycerol-3-phosphate (U)- $[^{14}\text{C}]$ to see if this precursor is incorporated into lysophosphatidylglycerol.

Table VI shows that $[^{14}\text{C}]$ -glycerophosphate does indeed incorporate into lysophosphatidylglycerol and unlabeled lysophosphatidylethanolamine stimulated this incorporation.

$[^{14}\text{C}]$ -glycerol did not so markedly incorporate into lysophosphatidylglycerol under similar conditions. However, ATP and Mg^{++} addition greatly stimulated the incorporation of $[^{14}\text{C}]$ -glycerol into lysophosphatidylglycerol.

These results indicate quite conclusively that lysophosphatidylglycerol formation occurs mainly via

TABLE VI

(A) The incubation mixture contained, in 1 ml., 0.1 M Tris buffer pH 7.0, 1.6 mg of particulate fraction protein, 5 mM CaCl_2 , 10 nmoles [^{14}C]glycerophosphate.

(B) The conditions are similar as stated for (A) except that [^{14}C]glycerophosphate was replaced by 15 nmoles of [^{14}C]glycerol.

(C) The conditions are the same as stated for (B) except that Mg^{++} (10 mM) and ATP (2.5 mM) were added.

TABLE VI

Incorporation of [¹⁴C]-glycerophosphate, [¹⁴C]-glycerol and the effect of lysophosphatidylethanolamine.

Conditions	Counts recovered in lysophosphatidylglycerol
(A) [¹⁴ C]-glycerophosphate +lysophosphatidylethanolamine (1 mg) (2 mg) (3 mg)	9 853 12 053 13 083 20 614
(B) [¹⁴ C]-glycerol +lysophosphatidylethanolamine (1 mg) (2 mg) (3 mg)	615 720 715 724
(C) [¹⁴ C]-glycerol + ATP + Mg ⁺⁺ +lysophosphatidylethanolamine (1 mg) (2 mg) (3 mg)	4 350 6 754 7 869 12 518

mechanism B.

To further prove this reaction, the lysophosphatidylglycerophosphate intermediate was systematically sought for, however it was not detected, very likely because of its rapid conversion to lysophosphatidylglycerol by the very active phosphatidylglycerophosphate phosphatase present in *Escherichia coli* (43). 20 mM fluoride did not cause accumulation of this intermediate, despite the inhibitory effect of fluoride on phosphatidylglycerophosphate phosphatase reported by Chang and Kennedy (43).

The effect of pH on the (^{14}C)-glycerophosphate incorporation was studied. Figure 8 indicates that the optimal pH range for the incorporation of (^{14}C)-glycerophosphate is similar but slightly lower than that found for the first reaction studied involving the conversion of (^{32}P)-lysophosphatidylethanolamine to labeled lysophosphatidylglycerol. This shift on optimum, could be due to unavoidable differences in assay medium. In initial studies with (^{32}P)-lysophosphatidylethanolamine the exogenous glycerophosphate concentration is high and that of added lysophosphatidylethanolamine, low. The reverse conditions held for studies implicating (^{14}C)-glycerophosphate incorporation.

FIGURE 8

The effect of pH on the incorporation of [^{14}C]-glycerophosphate (●—●) and [^{14}C]-ethanolamine (○---○) into lysophosphatidylglycerol and lysophosphatidylethanolamine. The assay mixture contained, in 1 ml, 13 nmoles [^{14}C]-glycerophosphate or 60 nmoles of [^{14}C]-ethanolamine, 1.5 mg cold lysophosphatidylethanolamine, 3.5 mg of particulate fraction protein. In the pH range 4-7, the buffer was 0.1 M acetate and in the pH range 7-9, 0.1 M Tris-HCL buffer was used.

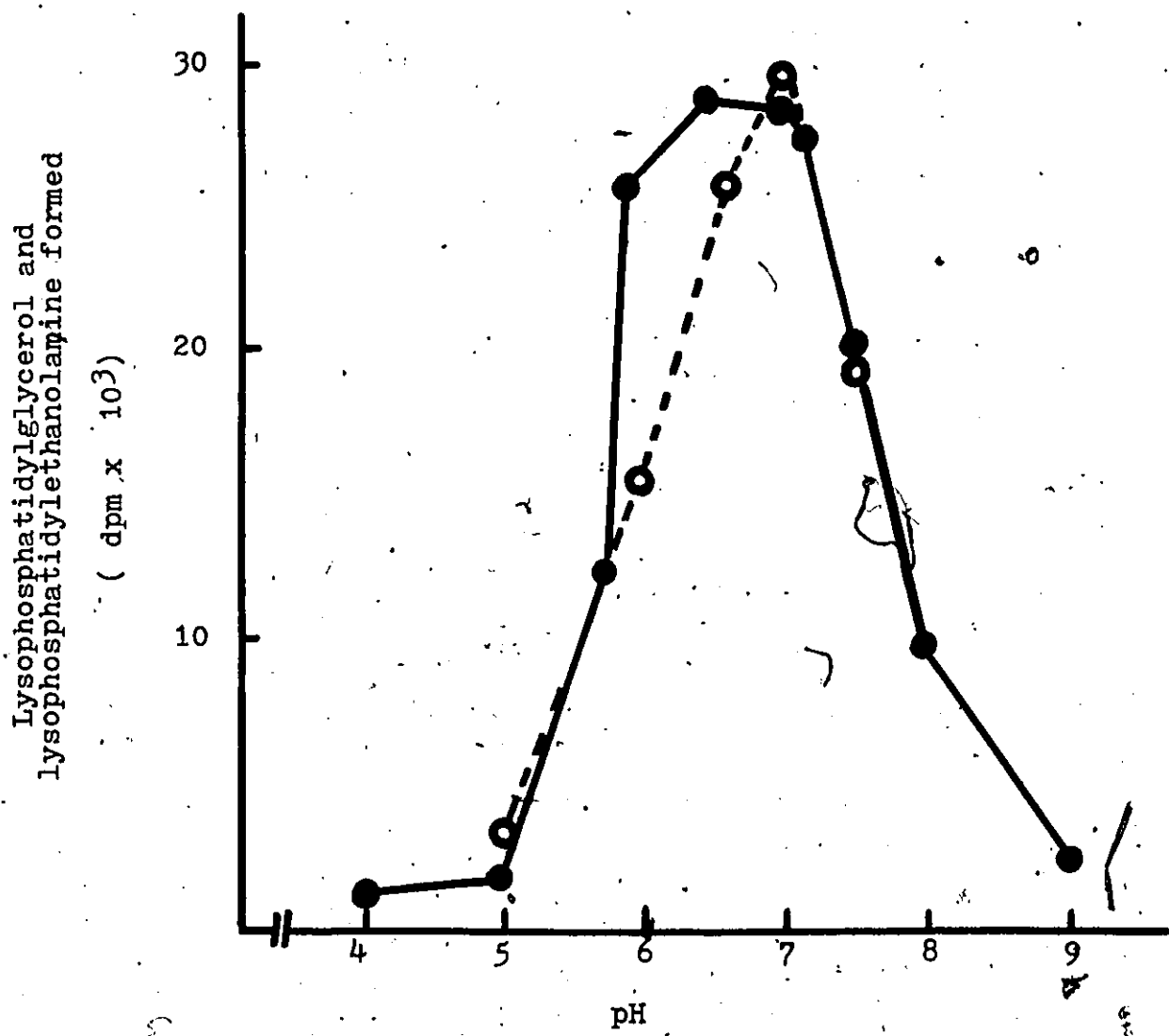


FIGURE 8

I.8. Studies to determine other possible exchange reactions.

I.8.1. A study of the incorporation of $[^{14}\text{C}]$ - ethanolamine into lysophosphoglycerides.

The Escherichia coli particulate fraction was incubated with $[^{14}\text{C}]$ - ethanolamine in the presence of Ca^{++} and the effect of unlabeled lysophosphatidylethanolamine, phosphatidylethanolamine and phosphatidylglycerol was studied. The results of this experiment summarized in Table VII indicate that $[^{14}\text{C}]$ - ethanolamine incorporates into lysophosphatidylethanolamine, the incorporation being stimulated by unlabeled lysophosphatidylethanolamine. Experiments in this laboratory have indicated that $[^{14}\text{C}]$ - ethanolamine incorporation displays a pH optimum of about 7.0 .

The addition of unlabeled lysophosphatidylglycerol did not stimulate the incorporation of $[^{14}\text{C}]$ - ethanolamine.

I.8.2. A study of the incorporation of $[^{14}\text{C}]$ - serine into lysophosphoglycerides.

$[^{14}\text{C}]$ - serine was incubated with Escherichia coli particulate fraction and Ca^{++} . The results summarized in

TABLE VII

(A) The incubation mixture contained, in 1 ml, 0.1 M Tris buffer pH 7.0, 5 mM CaCl₂, 1 mg of particulate fraction protein, 60 nmoles [¹⁴C]ethanolamine and various phospholipids as indicated.

(B) The incubation conditions are similar as those described for (A) except that [¹⁴C]ethanolamine was replaced by 39 nmoles of [¹⁴C]serine.

(C) The incubation conditions are the same as those described for (A) except that the [¹⁴C]ethanolamine was replaced by 15 nmoles of [¹⁴C]glycerophosphate.

TABLE VII

Studies to determine other possible exchange reactions.

		Counts recovered in.			
		LPE	LPG	PG	PE
(A)	[¹⁴ C]-ethanolamine	1 389			0
	+lysophosphatidylethanolamine	(0.5 mg)			0
		(1 mg)			100
		(2 mg)			128
		(1 mg)	27 346		100
(B)	+phosphatidylethanolamine	1 295			120
	+lysophosphatidylglycerol	(1 mg)			0
		(2 mg)			0
		(1 mg)	1 402		0
		(2 mg)	1 397		0
(C)	+phosphatidylglycerol	975			0
		(1 mg)			0
		(2 mg)	1 184		0
	[¹⁴ C]-serine				1 215
	+lysophosphatidylethanolamine	(1 mg)			1 114
(C)	+lysophosphatidylglycerol	(2 mg)			1 228
		(1 mg)			1 086
		(2 mg)			1 275
	[¹⁴ C]-glycerophosphate			3 784	180
	+phosphatidylglycerol	(1 mg)		3 520	0
(C)	+phosphatidylethanolamine	(2 mg)		3 075	0
		(1 mg)		2 615	0
		(2 mg)		2 509	0
		(1 mg)		2 104	0
		(2 mg)		1 915	0

Table VII, indicate that no incorporation of $[^{14}\text{C}]$ from $[^{14}\text{C}]$ -serine into lysophosphatidylethanolamine occurs even with the addition of unlabeled lysophosphatidylethanolamine or lysophosphatidylglycerol. Some $[^{14}\text{C}]$ from $[^{14}\text{C}]$ -serine was incorporated into phosphatidylethanolamine.

I.8.3. A study of the incorporation of $[^{14}\text{C}]$ -glycerophosphate in the presence of various exogenous lipids.

$[^{14}\text{C}]$ - glycerophosphate was incubated with Escherichia coli particulate fraction and Ca^{++} and the effect of unlabeled phosphatidylglycerol, lysophosphatidylglycerol, and phosphatidylethanolamine was studied. Under the incubation conditions used, glycerophosphate was incorporated only in lysophosphatidylglycerol and this incorporation is not stimulated by the addition of unlabeled phosphatidylglycerol, lysophosphatidylglycerol or phosphatidylethanolamine. Incorporation into phosphatidylglycerol was insignificant and was not increased by phosphatidylglycerol or phosphatidylethanolamine addition. These results (see Table VII) indicate that phosphatidylglycerol, lysophosphatidylglycerol and phosphatidylethanolamine do not exchange their bases with exogenous glycerophosphate.

Accordingly, one can conclude that under the varied substrate conditions stated, exchange reactions take

place only with lysophosphatidylethanolamine as lysophosphatidyl donor and glycerophosphate (possibly glycerol also) and ethanolamine as lysophosphatidyl acceptors.

Diacylphosphoglycerides do not apparently participate in exchange reactions, at least not in the absence of added nucleotides, with disrupted cells as enzyme source.

I.9. Exchange reactions in other bacteria.

Lysophosphoglyceride exchange reactions were sought in other bacteria.

Under the same conditions studied with Escherichia coli, lysophosphatidylglycerol formation occurred with Pseudomonas aeruginosa and Proteus vulgaris particulate fractions. These fractions were prepared in a manner identical to Escherichia coli particulate fraction.

The reaction with these other bacteria was also stimulated by Ca^{++} (see Table VIII).

The reaction did not occur when particulate fractions of Gram-positive bacteria such as Bacillus cereus, Bacillus subtilis and Staphylococcus aureus were used (see Table VIII).

TABLE VIII

The assay mixture contained in 1ml ,0.05 umol of [³²P]-lysophosphatidylethanolamine, 0.7 mg of particulate fraction protein of the corresponding bacteria, 0.1 M Tris buffer pH 7.3 and CaCl₂ as indicated.

TABLE VIII

Lysophosphatidylglycerol formation
in other bacteria.

Bacteria	Counts recovered in lysophosphatidylglycerol
Pseudomonas aeruginosa,	
No Ca ⁺⁺ added	688
Ca ⁺⁺ (5 mM)	2 470
Proteus vulgaris	
No Ca ⁺⁺ added	1 544
Ca ⁺⁺ (5 mM)	3 591
Bacillus cereus	
No Ca ⁺⁺ added	0
Ca ⁺⁺ (5 mM)	0
Bacillus subtilis	
No Ca ⁺⁺ added	0
Ca ⁺⁺ (5 mM)	0
Staphylococcus aureus	
No Ca ⁺⁺ added	0
Ca ⁺⁺ (5 mM)	0

GENERAL DISCUSSION

Although the pathways for phosphoglyceride synthesis in Escherichia coli have been elucidated for the most part by Kennedy's group and other workers (see reviews by Cronan (29) and Raetz (150)) the enzymes responsible for breakdown of these lipids are not completely characterized and their functions remain generally unknown.

Phosphatidylethanolamine, the main lipid of Escherichia coli does not turn over. However polyglycerophosphatides do turn over at a moderate rate by conversion of phosphatidylglycerol to cardiolipin and vice versa. These interconversions involve the polyglycerophosphatide cycle (50) comprising cardiolipin synthase and cardiolipin phosphohydrolase activities in vivo. They also turn over by donating an sn-glycero-1'-phosphate group to a certain class of oligosaccharides described by Van Golde et al. (40). Turnover of lipids does not seem to involve phospholipase A and lysophospholipase, well characterized enzymes of Escherichia coli. Interconversion between phosphatides other than between phosphatidylglycerol and cardiolipin had not been described prior to the present study.

I. The conversion of lysophosphatidylethanolamine to lysophosphatidylglycerol.

The results of this thesis demonstrate the presence in Escherichia coli particulate fraction of an enzymatic activity catalyzing base exchange reactions involving lysophosphatidylethanolamine as donor and glycerophosphate, glycerol and ethanolamine as acceptors of lysophosphatidyl groups.

The product of the reaction involving glycerophosphate, lysophosphatidylglycerol, was characterized as the intact lipid by co-chromatography with authentic 1-lysophosphatidylglycerol in several TLC systems and as the mild alkaline hydrolysis product, glycerophosphorylglycerol. Other analytical results obtained in this laboratory indicated that the intact product labeled with [^{32}P] could be degraded with phospholipase C to yield labeled glycerophosphate.

This product had been detected previously in our laboratory as material remaining at the origin when lysophospholipase activity had been tested in coliform extracts with [^{32}P]-labeled lysophosphatidylethanolamine. It was erroneously dismissed as water-soluble material contaminating the lipid phase. On other occasions the two lyso compounds

may not have clearly separated one from the other. With the more polar solvents described in this thesis, lysophosphatidylethanolamine moved at a faster rate and could be separated from lysophosphatidylglycerol which moved away from the origin and could be distinguished from any possible water-soluble contaminants.

II. Factors affecting the formation of lysophosphatidylglycerol.

Ca^{++} at concentrations below 10 mM had a stimulatory effect but addition of this cation was not required for activity. Since EDTA inhibited the reaction completely, it is likely that the enzyme does require Ca^{++} . At higher concentrations, Ca^{++} was inhibitory. Mg^{++} , Ca^{++} , Cd^{++} and Mn^{++} were inhibitory even at lower concentrations.

Preliminary experiments (results not shown) indicated that the reaction was not enhanced in the presence of ether or detergents.

The conversion of (^{32}P) -lysophosphatidylethanolamine to labeled lysophosphatidylglycerol occurred optimally between pH 6.8-8.0. The incorporation of sn-glycero-3-phosphate (U)- (^{14}C) into labeled lysophosphatidylglycerol had a slightly lower pH optimum range (6.5 - 7.5) but this difference could be explained by the slight difference in conditions

as explained in the previous section.

The incorporation of sn-glycero-3-phosphate (U) [^{14}C] was significantly stimulated by addition of unlabeled lysophosphatidylethanolamine and the conversion of lysophosphatidylethanolamine to lysophosphatidylglycerol was enhanced by addition of DL- α -glycerophosphate. These results are all indicative of an exchange reaction involving the base part of the lipid substrate.

Like the coliform exchange reaction just described, base exchange reactions in mammalian tissues, localized in particulate fractions, are generally characterized as being stimulated by Ca^{++} . However, they have a rather alkaline pH optimum (136, 137, 151, 152).

III. The mechanism of lysophosphatidylglycerol formation.

When doubly-labeled lysophosphatidylethanolamine was used as substrate, lysophosphatidylglycerol formation occurred without a change in the $^{14}\text{C}/^{32}\text{P}$ ratio (see Table VI), indicating that the lysophosphatidyl group was transferred intact to form the product. Since glycerophosphate markedly stimulated lysophosphatidylglycerol formation in contrast to glycerol which did so only very slightly and since the effect of glycerol and glycerol (U) [^{14}C] was in turn increased by ATP addition, one can conclude that glycerophosphate is the

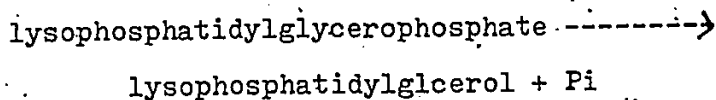
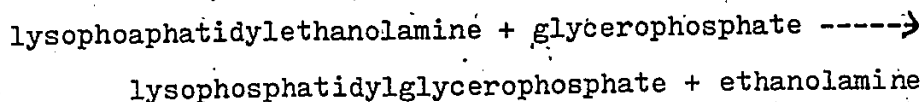
preferred acceptor of the lysophosphatidyl group for the formation of lysophosphatidylglycerol.

These results do not preclude a mechanism directly involving glycerol as lysophosphatidyl acceptor. Perhaps another, less specific enzyme also catalyzing exchange with ethanolamine is involved in this case. The enhancing effect of Mg^{++} and ATP was unexpected since Mg^{++} normally inhibits lysophosphatidylethanolamine conversion to lysophosphatidylglycerol. On the other hand, since Ca^{++} addition to the incubation medium is not strictly required for activity, some of the endogenous Ca^{++} must remain tenaciously bound to the particulate enzyme, even after washing or dialysis and could partially protect against the effect of Mg^{++} .

It is likely that lysophosphatidylglycerol synthesis occurs with the formation of lysophosphatidylglycerophosphate as intermediate. This product could not be detected likely because it is rapidly hydrolyzed to yield lysophosphatidylglycerol. An analogous situation exists in the case of phosphatidylglycerol formation. Phosphatidylglycerophosphate, the precursor of phosphatidylglycerol, does not accumulate because it is rapidly hydrolyzed by a very active phosphohydrolase. Fluoride (20 mM) was reported to inhibit the phosphatidylglycerol phosphohydrolase of *Escherichia coli*. However, addition

of this ion to the incubation medium did not cause accumulation of lysophosphatidylglycerophosphate. The reason for this negative result is not clear.

The mechanism which we postulate for lysophosphatidylglycerol formation is illustrated in the following reactions:



The enzyme involved appears to be quite different from plant phospholipase D or from cardiolipin phosphodiesterase since the lysophosphatidyl group is always transferred to an alcohol (glycerophosphate) and not only to water. It appears to be a true transphosphatidylase enzyme of narrow specificity.

Experiments in our laboratory have indicated that when lysophosphatidyl [^{14}C]- ethanolamine was incubated with Escherichia coli particulate fraction in the presence of Ca^{++} , [^{14}C]- ethanolamine was recovered in the water-methanol phase after Bligh and Dyer extraction, but this product could have arisen partly by the breakdown of

[¹⁴C]- glycerophosphorylethanolamine formed by a lysophospholipase action on lysophosphatidylethanolamine. The [¹⁴C]- ethanolamine recovered, sometimes increased with the addition of glycerol and usually more so with glycerophosphate added to the incubation medium (data not shown). But concurrent and variable breakdown of [¹⁴C]- glycerophosphorylethanolamine under the conditions of assay interfered with a proper assessment of [¹⁴C]- ethanolamine produced by transphosphatidylase. This experiment might be advantageously repeated in the presence of deoxycholate which is known to inhibit lysophospholipase (98) however it is not certain what effect this detergent might have on the transphosphatidylase enzyme.

The sequence of reactions described above would lead to the formation of a product of the correct stereochemical configuration, namely, sn-1-acyl glycerol-3-phosphoryl sn-1'-glycerol. (This is the stereochemical configuration found for phosphatidylglycerol of *Escherichia coli*). This point could be clarified provided enough of the product can be isolated. The configuration of the acylated and unacylated glycerol can be estimated by hydrolyzing the product with NaOH, correcting for any β - glycerophosphate formed and assessing the yield of sn-glycerol-3-phosphate with stereospecific sn-glycerol-3-phosphate dehydrogenase. When only half of the total β - glycerophosphate is used up by the enzyme then this means that the product is indeed

a derivative of sn-glycero-3-phosphoryl-sn-1'-glycerol. On the other hand if a transphosphatidylation occurred directly with glycerol one would expect the product to be a derivative of sn-glycero-3-phosphoryl sn-1' (3') glycerol if the enzyme involved behaves like plant phospholipase D (153). Indeed when phosphatidylcholine is incubated with 4% glycerol and cabbage leaf phospholipase D, a phosphatidyl rac-1'(3') glycerol product is obtained (153).

IV. Other exchange reactions.

Ethanolamine was also found to be an acceptor of the lysophosphatidyl group of lysophosphatidylethanolamine. The reaction was not characterized in detail however. Indicative of this type of reaction, the incorporation of [^{14}C] ethanolamine into lysophosphatidylethanolamine was stimulated by addition of unlabeled lysophosphatidylethanolamine to the reaction mixture. Results in our laboratory have also shown that this reaction is Ca^{++} requiring and has a pH optimum between 7.0 - 7.5. Serine- [^{14}C] did not incorporate into lysophosphatidylserine or lysophosphatidylethanolamine which means that this exchange reaction is specific and could well involve a different enzyme than the one catalyzing lysophosphatidylglycerol formation.

Exchange reactions involving lysophosphatidyl-glycerol as lysophosphatidyl donor were not detected. Furthermore, addition of phosphatidylethanolamine or phosphatidylglycerol to the incubation mixture did not cause enhanced incorporations of sn-glycero-3-phosphate- $[^{14}\text{C}]$, serine- $[^{14}\text{C}]$ or ethanolamine- $[^{14}\text{C}]$ into any of the phosphatide fractions. Hence no evidence for other exchange reactions could be obtained.

V. The possible physiological significance of the lysophosphoatidylethanolamine exchange reactions.

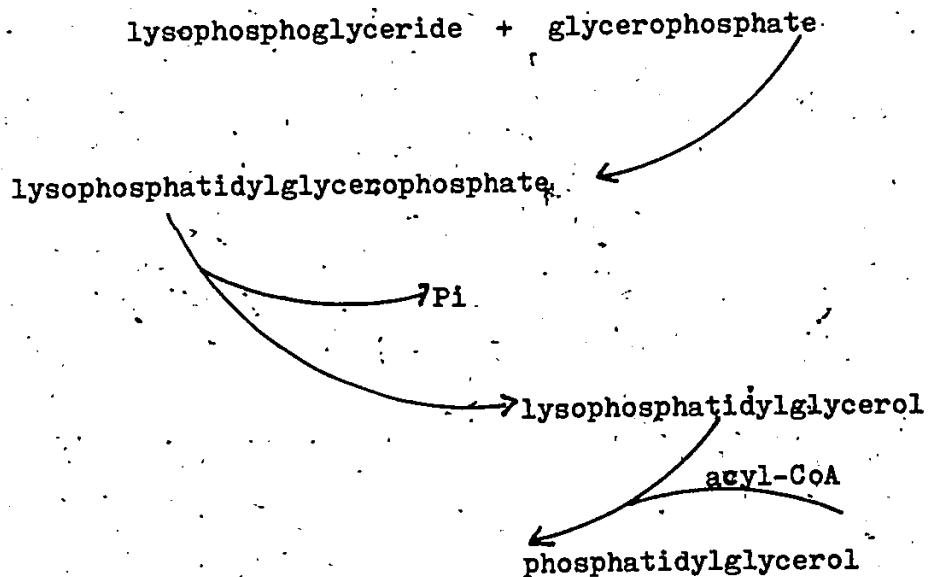
The enzymes for the deacylation-reacylation cycle occur in Escherichia coli. These are, the lysophosphoglyceride acylating enzyme, (121, 154) and phospholipase A (114, 116, 121) catalyzing the reactions illustrated below :

- (1) phosphoglyceride $\xrightarrow{\text{phospholipase A}}$ fatty acid + lysophosphoglyceride
- (2) lysophosphoglyceride $\xrightarrow{\text{acylation}}$ phosphoglyceride (2 acyl)

Although the presence of phospholipase A has been demonstrated in Escherichia coli, involvement of its activity in phosphoglyceride turnover cannot be ascertained (155). Thus, the deacylation-reacylation cycle may not be

functional as such. However one could picture the involvement of this cycle in localized areas of the membrane which would escape detection by the usual pulse-chase type of experiments.

Phospholipase A is located in the outer membrane fraction (81) and is thus well suited for degrading exogenous phosphoglycerides. Exogenous phosphatidylethanolamine could be hydrolyzed to yield lysophosphatidylethanolamine, which through the exchange reaction would be converted into lysophosphatidyl sn-1'-glycerol (sequence below). This product, of the correct configuration, could in turn be acylated (reaction 2) to yield phosphatidylglycerol.



Thus the transphosphatidylation reaction could be pictured as having a homeostatic function, serving to maintain the relative proportion of phosphatidylglycerol as exogenous lipids incorporate into the membrane of Escherichia coli and other Gram-negative bacteria. It would be interesting to see if the transphosphatidylation enzyme can also convert lysophosphatidylcholine to lysophosphatidylglycerol. Since the polar head group influences the fluidity of phosphoglycerides, it is indeed important that the membrane be equipped with means to keep the relative proportions of phospholipid classes constant. To date no mechanisms have been proposed to account for the rigid control of the relative amount of phosphatidylethanolamine (70-75% of the total phospholipid) and polyglycerphosphatides (20-30% of the total phospholipid). An exchange mechanism occurring directly between phosphatidylethanolamine and phosphatidylglycerol could serve in such a regulatory function and would likely be under the control of simple factors such as availability of glycerophosphate for example. However, exchange reactions were found not to occur with diacyl phosphoglycerides at least under the experimental conditions tested.

Since phospholipase A is inactive against endogenous lipids in normal cells, it is unlikely that exchange reactions play a role in controlling the proportions of endogenously derived lipids. However the de novo formation

of lysophosphoglycerides in *Escherichia coli* has never been precluded. But if this occurred it is possible that some control mediated by exchange reactions at the lysophosphoglyceride level occurs.

Ballesta et al. (149) suggested that the incorporation of glycerol into phosphatidylglycerol in mutants incapable of synthesizing glycerophosphatide occurred via a direct exchange reaction. We were unable to obtain evidence for such a reaction, however the conditions for testing exchange between glycerol and phosphatidylglycerol were not extensively varied. On the other hand, exchange of glycerol with the unacylated glycerol moiety of phosphatidylglycerol could arise indirectly by reverse of the cardiolipin synthase reaction (50):



Serine synthetase is known to catalyze exchange of serine in phosphatidylserine. This particular reaction was not verified with added serine and added phosphatidylserine under our conditions of incubation. Some serine did incorporate into phosphatidylethanolamine, likely via formation of phosphatidylserine and its decarboxylation, when only Ca^{++} was added to the medium however this incorporation was not stimulated by addition of phosphatidylethanolamine. Serine incorporation may have been assisted by endogenous cofactors leading to de novo synthesis of phosphatidylserine, however a simple exchange reaction may have been involved as well whereby serine- ^{14}C replaces serine in endogenous phosphatidylserine.

In conclusion, one may add that the rather restricted number of exchange reactions that do occur in Escherichia coli seems to point to the involvement of rather specific enzymes and since the enzymes are specific, they must play specific roles. At the moment we can only conjecture these roles, however, mutants may be isolated eventually which lack these base exchange enzymes. The phenotypes displayed may then reveal these functions more clearly.

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