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Studies on Phosphatidylethanolamine Synthesis in *Escherichia coli*

by

Cheryl L. Tomalty

A Thesis

Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Department of Biochemistry
University of Ottawa

June 1979

C.L. Tomalty, Ottawa, Canada, 1979

C.L. Tomalty, Ottawa, Canada, 1979.

To my Mother and Father

who have given me their love

and support.

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LIST OF ABBREVIATIONS

PA	Phosphatidic Acid
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
CL	Cardiolipin
PS	Phosphatidylserine
PC	Phosphatidylcholine
GP	Glycerophosphate
GPG	Glycerophosphorylglycerol
GPE	Glycerophosphorylethanolamine
GPGPG	Glycero-(diphosphoryl)-glycerol
PGP	Phosphatidylglycerophosphate
LPS	Lipopolysaccharide
MAH	Mild Alkaline Hydrolysis
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced)
ACP	Acyl Carrier Protein
CoA	Coenzyme A
RNA	Ribonucleic Acid
AMP	Adenosine Monophosphate
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CMP	Cytidine Monophosphate
CDP	Cytidine Diphosphate
dCDP	Deoxycytidine Diphosphate

CTP	Cytidine Triphosphate
UTP	Uridine Triphosphate
GTP	Guanosine Triphosphate
ppGpp	Guanosine 5'-diphosphate-3'-diphosphate
SDS	Sodium Dodecyl Sulphate
EDTA	Ethylenediaminetetraacetic acid
KDO	2-Keto-3-Deoxyoctonate

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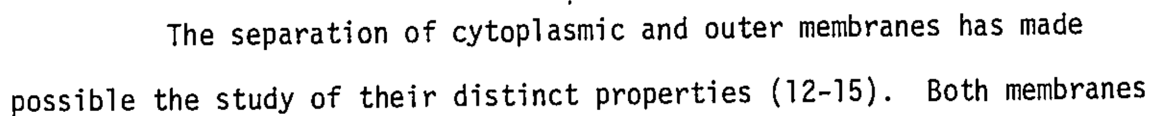
INTRODUCTION

A. General Characteristics of the Gram-Negative Cell Envelope

Electron microscopic studies have shown that the cell envelope of Gram-negative bacteria is composed of two distinct membranes, the inner cytoplasmic membrane and the outer membrane, both of which are known to contain lipid bilayers (1,2). The inner membrane is the site of several vital functions including respiration and ATP production. It is the primary permeability barrier of the cell and contains specific transport systems (3,4,5). It is also the site where the peptidoglycan units are assembled and where most of the synthesis of phosphoglycerides occurs (3,6,7). The outer membrane has other characteristic but less vital functions. Its lipopolysaccharide and protein components which are exposed to the exterior have typical antigenic and binding properties (3,8,9). The outer membrane also serves as a passive barrier to substances of molecular weight over several hundred and this explains in part why Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria lacking this structure (10,11).

A rigid peptidoglycan is located between the two membranes in an area designated as the periplasmic space. This space also accommodates hydrolytic enzymes and other soluble binding proteins involved in transport and chemotaxis (3,4,5).



The separation of cytoplasmic and outer membranes has made possible the study of their distinct properties (12-15). Both membranes

consist of approximately equal amounts of proteins and lipids, but there is an asymmetric distribution of the proteins within each membrane (3,13,16).

The outer membrane contains four major proteins which have been designated matrix proteins 1a + 1b, protein 2, TolG protein, and protein 3b (17). Matrix proteins 1a and 1b are strongly associated with the peptidoglycan. The TolG protein is characterized as heat-modifiable and trypsin-sensitive. Proteins 2 and 3b have not yet been well characterized (17). In addition to these major proteins, there are 10-20 minor proteins present in the outer membrane. The major proteins along with some minor ones appear to function as receptors for bacteriophages and colicins. A few are involved in the processes of conjugation and cell division, others are concerned with specific uptake systems for nutrients such as iron, vitamins, and carbohydrates, and in specific facilitated diffusion of low-molecular weight solutes (17).

The outer membrane also contains a major lipoprotein which is covalently linked to the peptidoglycan layer. This lipoprotein has a diglyceride residue attached to the N-terminal cysteine. Its fatty acid composition reflects that of the phosphoglycerides of the envelope and must be derived from one of these lipids by a mechanism yet unknown (18).

Gram-negative bacteria characteristically contain lipopolysaccharide in the outer membrane which is not found in any Gram-positive organisms. The lipopolysaccharide occupies about 45% of the surface of the outer membrane. Its structure as illustrated in Figure 1 is composed of an outermost region which is the O antigen containing the typical antigenic

```
                    col              glcNAc                                    [glc] *                                P
                     |α1-4            |α1-2                                       |                                  /
[colα1-6 glcNAcβ1-2 glcα1-2 gal]  -glcα1-2 galα1-3 glc [glcN] * hepα1-3 hepα1-?KDO(KDO,KDO)-glcN1-6 glcN
                                n                               \_____________/                     \_________/
                                                                 ethanolamine                       6 fatty acids
                                                                      :                            /
                                                                      P                           P

|______________________________| |___________________________________________________________| |____________|
      antigenic side chain                                core                                      lipid A
```

Figure 1

Probable Structure of Lipopolysaccharide

of *E. coli*. (10)

* These residues exist on only some of the core molecules

Abbreviations: col - colitose; glcNAc - N-acetylglucosamine; glc - glucose; gal - galactose; glcN - glucosamine; hep - heptose; KDO - ketodeoxyoctulonate. The nature of the bond to KDO is not known. Two classes of molecules where n=1 and n=9 or 10 exist in the wild-type organism.

polysaccharide material giving rise to the serological classes of coliform organisms, an outer and inner core region, and an innermost region, lipid A (4,10,19). Pyrophosphoethanolamine moieties are covalently linked to heptose and/or KDO units in the core portion (20). Lipid A contains glucosamine, the fatty acids laurate, myristate, and β-hydroxymyristate, and esterified phosphate (10). From the lipid A portion buried in the outer membrane, the polysaccharide chains extend outward into the environment. Since lipid A replaces phosphoglycerides in the outer half of the bilayer, the inner half contains more phosphatides (21).

Approximately 40% of the total phospholipid of *E. coli* is located in the inner membrane, the rest is in the outer membrane. No phospholipid is normally found in the cell cytosol nor in the culture medium (13). The phosphoglyceride compositions of the inner membrane and the outer membranes are similar although the phospholipid content per milligram of protein is, in the inner membrane, almost twice that in the outer membrane (22).

Synthesis of all components of the Gram-negative envelope takes place in the cytoplasmic membrane from precursor molecules derived from the cytoplasm (13,23). These processes include most of the steps in the formation of phosphoglycerides and many in the formation of peptidoglycan units. The products are then translocated across the inner membrane and are deposited in the peptidoglycan and the outer membrane regions by mechanisms not yet fully understood (24,25,26).

Like all other membranes studied, the inner and outer membranes of *E. coli* display asymmetry in the distribution of lipid and protein in a plane perpendicular to the surface of the cell (24,25,26).

B. Fatty Acids of *E. coli*

(i) Occurrence

All but very small amounts of the fatty acids of *E. coli* are covalently linked to phosphoglycerides. Only trace quantities can be detected in the free form. Of the saturated species, palmitic acid is the most abundant, comprising 25-40% of the total fatty acids in *E. coli*. Minor saturated analogues include stearic acid (approximately 1%), lauric acid (approximately 1%), myristic acid (1-5%) and the cyclopropane fatty acids; cis-9,10- methylene hexadecanoic acid (1-20%) and cis-11,12- methylene octadecanoic acid (approximately 1%). The unsaturated species are all of the cis configuration and contain only one double bond (24,25). These include palmitoleic acid (approximately 25-40%) and cis-vaccenic acid (approximately 25-37%) (26,27,28).

The proportions of the different fatty acids vary according to the conditions of growth, the nutrients available, and the age of the culture (24,25,29-32). Low temperatures favor a higher percentage of unsaturated species whereas aging causes an accumulation of cycloproprane fatty acids (29,30).

(ii) Metabolism

The pathway for the formation of fatty acids is shown in Scheme 1. The steps involved have been amply discussed in recent reviews to which the reader is referred (33,34). The synthesis of fatty acids in *E. coli* does not take place on an enzyme complex as it does in eucaryotic systems although similar steps are involved.

Acetyl-S-CoA + ACP-SH → Acetyl-S-ACP ; → CoA-SH

Malonyl-SCoA + ACP-SH

↓

Malonyl-S-ACP

Acetyl-S-ACP → Acetyl-S-Synthase

Synthase-H → Acetyl-S-Synthase

Malonyl-S-ACP + Acetyl-S-Synthase → Acetoacetyl-S-ACP

↓

CH_3-CHOHCH$_2$CO-S-ACP

↓

CH_3-C(H) = (H)C-CO-S-ACP

↓

$CH_3CH_2CH_2$CO-S-ACP - - - 3 cycles - - - → $CH_3(CH_2)_5CH_2$CH(OH)-CH$_2$C(=O)-S-ACP

β,γ-dehydrase → $CH_3(CH_2)_5$-C(H) = C(H)CH$_2$C(=O)-S-ACP - - → PALMITOLEATE and cis-VACCENATE

α,β-dehydrase → $CH_3(CH_2)_5$ CH$_2$C(H)=C(H)C(=O)-S-ACP - - Reduction 3 cycles elongation - - → PALMITATE

Scheme 1

Pathways for the Synthesis of Fatty Acids

It is interesting to note also that the formation of unsaturated fatty acids in *E. coli* involves a de novo process rather than the desaturation of preexisting saturated analogues as occurs in mammalian systems (33,34). This process involves the same array of enzymes as that concerned with the synthesis of saturated anologues except at one step involving the hydroxydecanoyl derivative which is the branch point leading either to the fully saturated palmitic acid or to unsaturated palmitoleic and cis-vaccenic acids. A β,γ dehydrase assures the formation of unsaturated species whereas an α,β dehydrase mediates the formation of saturated species (33,34,35).

Fatty acids are elongated in *E. coli* as thioester derivatives of acyl carrier protein (33,34). Exogenously supplied fatty acids can be activated to form thioesters either with coenzyme A or with acyl carrier protein (36-39). Two separate activating enzymes seem to be involved (38).

The cyclopropane derivatives of palmitoleate and cis-vaccenate are produced mostly during the stationary phase. The cyclopropane ring is formed by a membrane-bound enzyme which adds the methyl group of S-adenosylmethionine to the double bond of the precursor fatty acid esterified to the phospholipid (40,41,42).

E. coli has all the eucaryotic equipment to degrade fatty acids but the enzymes involved in the β-oxidation process are inducible (43).

C. Phosphoglycerides of *E. coli*

(i) Occurrence and Functions

In *E. coli*, the function of the phosphoglycerides is mainly structural: all three main phosphatides, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin are implicated in the lipid bilayer structure of the membrane. The structures of these phospholipids are given in Figure 2. These lipids are also specifically involved in the biological function of certain membrane proteins.

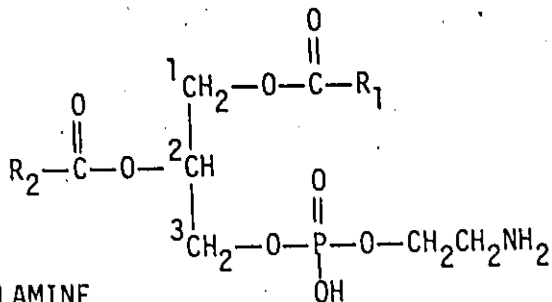
Phosphatidylethanolamine is the most abundant phospholipid, corresponding to approximately 70-80% of the total lipid of the cell (28,44,46). In addition to its structural role, this lipid is involved in the synthesis of lipopolysaccharide material: the enzymes concerned with the formation of the core polysaccharides are activated by phosphatidylethanolamine (47). This lipid is not of vital importance to the cell since mutants incapable of its synthesis grow normally (48).

Phosphatidylglycerol comprises 5-15% of the total *E. coli* lipid. It is implicated in the transport of monosaccharides (48,49), the formation of lysophosphatidic acid (50), and the activation of respiratory carriers (51). It is the precursor of cardiolipin (52,53), and likely the precursor of several classes of glycerophosphate-containing oligosaccharides (54,55).

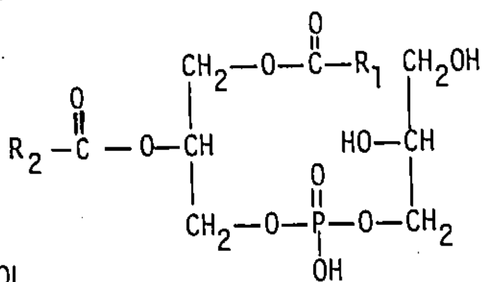
Cardiolipin accounts for about 5-15% of the phospholipids of *E. coli* (35). The role of this lipid is structural mainly but there is some evidence suggesting its activation of electron carriers (53). Levels of cardiolipin increase at the expense of phosphatidylglycerol when cells are aged or starved (56).

Figure 2

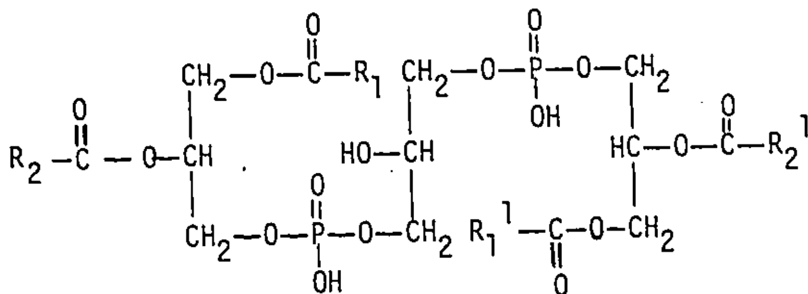
Structure of the Major Phospholipids of *E. coli*



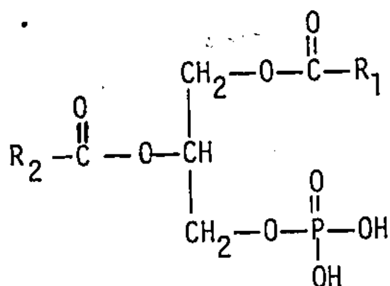
PHOSPHATIDYLETHANOLAMINE



PHOSPHATIDYLGLYCEROL



CARDIOLIPIN



PHOSPHATIDIC ACID

Most of the other minor phosphoglycerides of *E. coli*, phosphatidic acid, CDP-diglyceride, phosphatidylserine, and phosphatidylglycerophosphate, are involved as intermediates in the synthesis of the main species.

(ii) Synthesis of Phosphoglycerides

(a) General Comments

The steps leading to the formation of phosphatidylethanolamine and the polyglycerophosphatides are summarized in Scheme 2. The synthesis involves the successive acylation of positions 1 and 2 of *sn*-glycero-3-phosphate and the activation of the phosphatidic acid formed to yield CDP-diglyceride. At this point the pathway branches to form phosphatidylserine and phosphatidylglycerophosphate which can give rise to the main phosphatide classes discussed. In *E. coli* the evidence reported thus far does not suggest the involvement of an interaction of diglyceride with an activated hydroxy compound such as CDP-choline or CDP-ethanolamine as is found in animal cells (cf. Scheme 3).

There have been three different experimental approaches to elucidate the pathway of phospholipid biosynthesis shown in Scheme 2. The first approach involving pulse radioactive labelling of whole cells followed by a chase demonstrated the existence and rapid turnover of intermediates such as phosphatidic acid, CDP-diglyceride, and phosphatidylserine (57-59) and kinetics of

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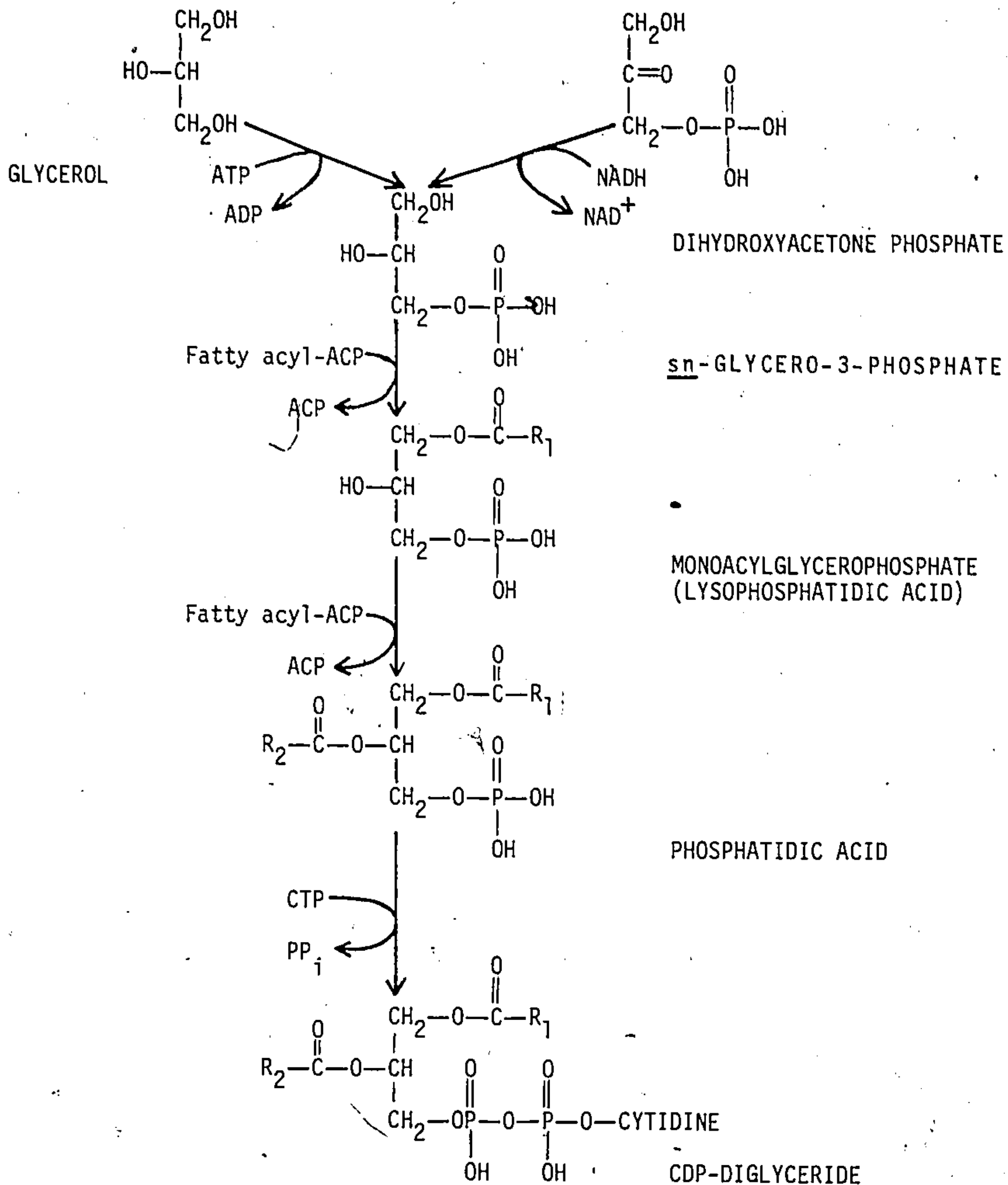
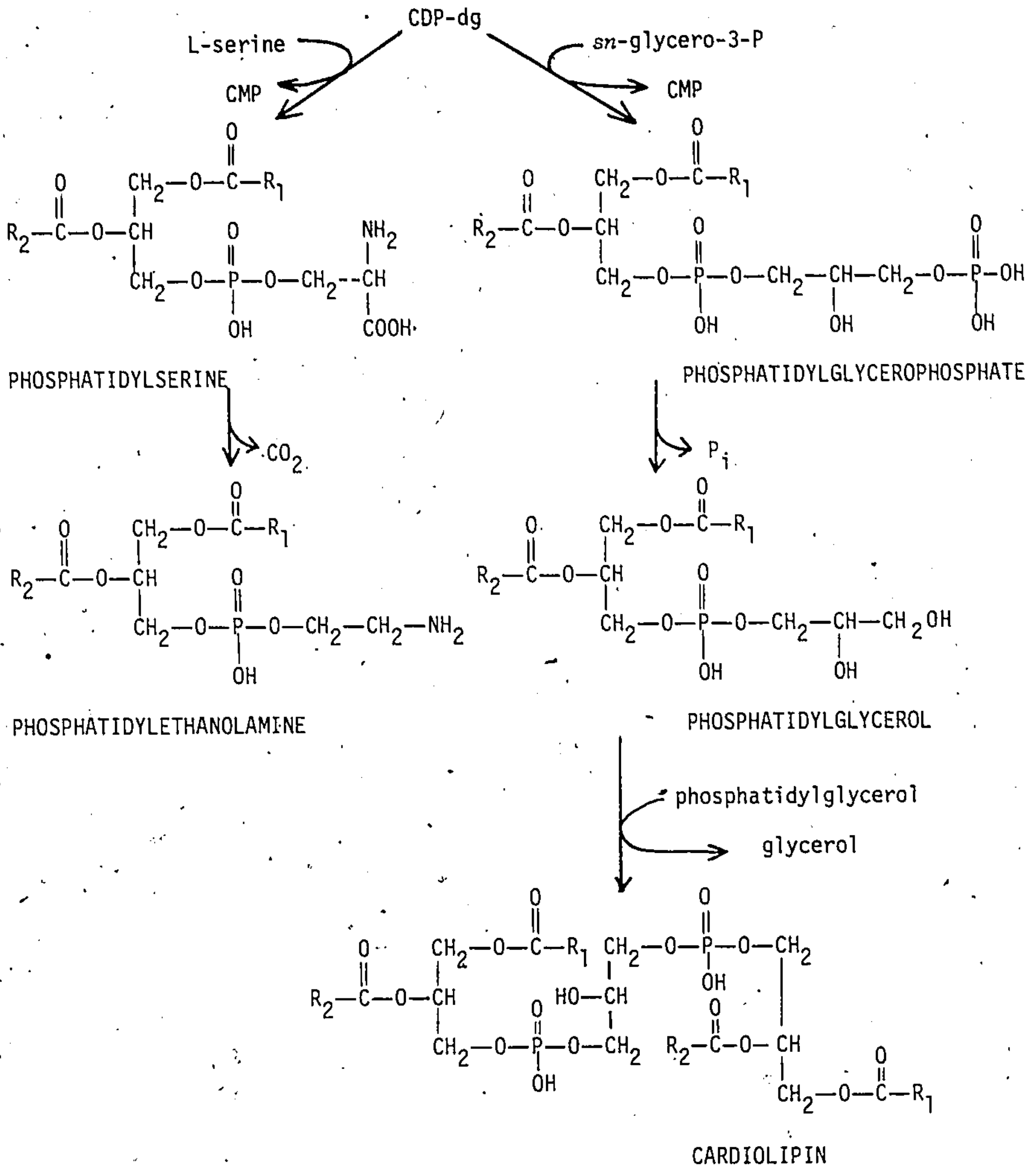
Scheme 2

Biosynthesis of Phospholipids in *E. coli*

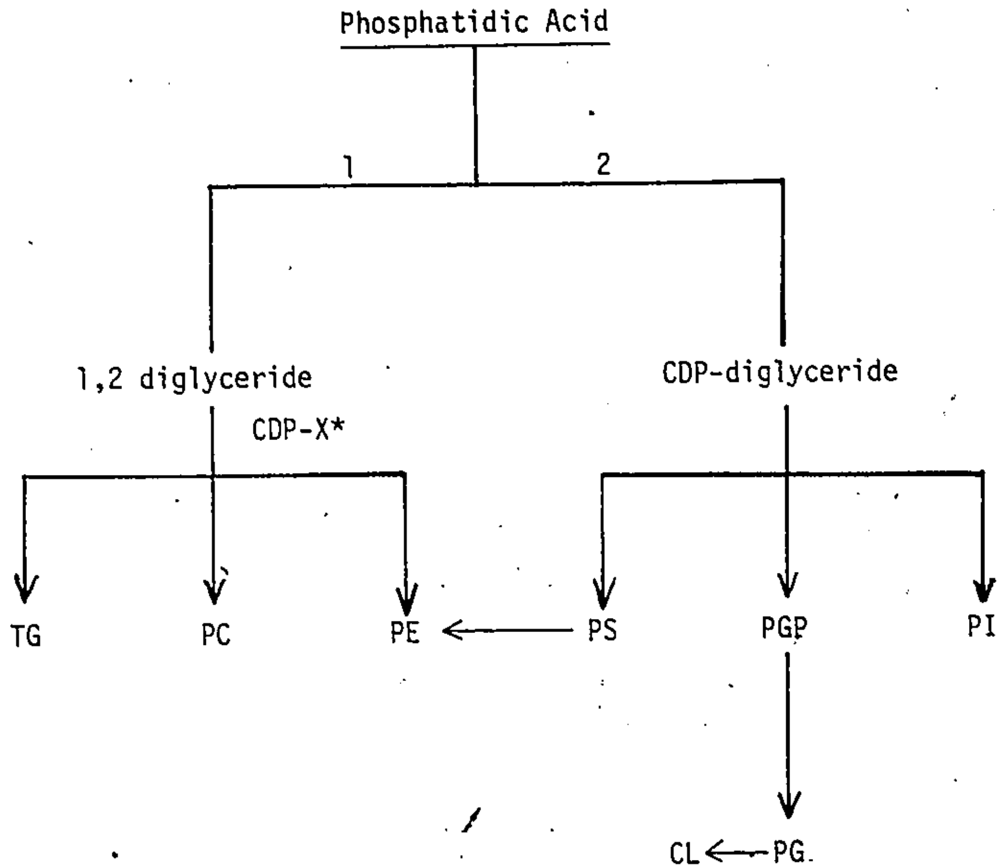


Scheme 2 (continued)



Scheme 3

Major Reactions in Mammalian Phospholipid Biosynthesis



Abbreviations: TG, triglyceride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PGP, phosphatidylglycerophosphate; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin.

* X, choline or ethanolamine.

labelling of the different lipids which were compatible with the given Scheme.

The second approach used by Kennedy and coworkers (52,60-63) demonstrated that the enzyme in crude cell extracts could catalyze the reactions of the pathway when exogenous labelled precursors were used as substrates. A number of these enzymes were subsequently purified and well characterized.

The third approach has concerned the isolation of mutants lacking certain enzymes of the alleged pathway. This approach is fairly recent and has offered some insights into the importance of certain enzymes for the synthesis and turnover of *E. coli* lipids and has begun probing the possible control points of phospholipid biosynthesis which have not been very well understood up to now.

All of the biosynthetic steps occur in the inner membrane except for the formation of phosphatidylserine by phosphatidylserine synthetase, which after homogenization of the cell is found tightly bound to the ribosomes (58,64,65).

(b) Synthesis of Phosphatidic Acid

1. Acylation of *sn*-glycero-3-phosphate

The synthesis of phosphatidic acid results from the two-step acylation of *sn*-glycero-3-phosphate. This water-soluble precursor is formed in the cytosol from dihydroxyacetone phosphate by a dehydrogenase using either NADH or NADPH (66).

It has now been shown with mutants lacking this enzyme (*gps* A mutants) that new membrane phospholipid synthesis can be stopped if exogenous *sn*-glycerol-3-phosphate is not supplied. The formation of *sn*-glycerol-3-phosphate from glycerol and ATP is not an effective route in *E. coli* since glycerol kinase is inhibited by low concentrations of fructose-1, 6-diphosphate (67-69).

The acylation of *sn*-glycero-3-phosphate to phosphatidic acid takes place by involving two different enzymes. This was demonstrated with mutants of *E. coli* containing a thermolabile glycero-3-phosphate acyltransferase and a thermoresistant monoacylglycerophosphate acyltransferase (70).

In *E. coli*, the saturated fatty acids occupy mainly position 1 and the unsaturated fatty acids mainly position 2 of the glycerophosphate backbone. Because of some measure of specificity of the acylating enzymes toward the fatty acid substrate, acylation of *sn*-glycerol-3-phosphate has been suggested to be the step at which the positional fatty acid composition of the phosphoglycerides is determined. 1-Palmitoyl-*sn*-glycero-3-phosphate was formed with palmitoyl CoA as acyl donor while 2-acyl-*sn*-glycero-3-phosphate was synthesized with palmitoleoyl-, oleoyl-, or cis-vaccenoyl-CoA as acyl donors (70). The same result was obtained when acylation was achieved with a mixture of saturated and unsaturated acyl-CoA derivatives (71). However apparently conflicting evidence (72-76) has shown

that both saturated and unsaturated fatty acids can be incorporated into 1-acyl-*sn*-glycero-3-phosphate depending on the conditions. Thus it would seem that the positional specificity of the acyltransferases is not rigid. Formation of position-specific phosphoglycerides could then be explained on the basis of different rates of acylation with saturated and unsaturated CoA derivatives and in accordance with this postulation, there are data which indicate that acylation of glycerophosphate proceeds faster with saturated CoA derivatives (71,74,76,77) whereas the incorporation of the second acyl group on the 1-acyl glycerophosphate known to be the intermediate in the synthesis of phosphatidic acid (74) is faster with an unsaturated acyl-CoA derivative (74).

With cell extracts, the acylation of *sn*-glycero-3-phosphate may be achieved with either acyl-CoA or acyl-ACP with different specificities (76,78). In vivo, it is not clear which of the two activated fatty acids serve as substrates. Mutants deprived of acyl-CoA synthetase can incorporate endogenous but not exogenous fatty acids into phosphoglycerides (36).

There is an enzyme in *E. coli* which can convert fatty acid to acyl-ACP (37). The function of this enzyme is unknown but it is possible that it serves to convert exogenously derived fatty acids to acyl-ACP derivatives which in turn would incorporate the acyl groups into phosphoglycerides (37).

2. Diglyceride Phosphokinase

A diglyceride kinase capable of forming phosphatidic acid from diglyceride and ATP in cell extracts, first reported by Pieringer and Kunnes (54), has been purified 600-fold. It is specific for the *sn*-1,2 isomers, but catalyzes the phosphorylation of monoglycerides and ceramides (79,80).

It is not possible to assign an important role to this enzyme in the synthesis of phosphatidic acid since in normal strains, diglyceride is a very minor pool of lipid and does not turn over rapidly (57,81) as compared to phosphatidic acid and CDP-diglyceride (57,59). Mutants which lack diglyceride kinase have been isolated and the membranes of these strains were found to contain substantial amounts of 1,2 diglyceride, representing approximately 8% of the total lipid (81). The phospholipid content of these mutants, however, is not altered. On the basis of these results it has been suggested that the diglyceride phosphokinase pathway functions as a minor route for phosphatidic acid synthesis.

(c) Synthesis of CDP-diglyceride

The formation of CDP-diglyceride involves the enzyme CTP: phosphatidic acid cytidyltransferase, an enzyme originally discovered in animal tissues (82). In *E. coli* the enzyme is associated with the inner membrane (6,7) and has been purified 100-fold by digitonin extraction and separation on DEAE cellulose (83). The synthetase

specifically requires *sn*-phosphatidic acid containing unsaturated fatty acids and is inactive with dipalmitoyl phosphatidic acid. It is dependent on the presence of Mg^{++} and K^{+} for maximal activity (60).

Results obtained with partially purified enzymes indicate that very likely a single enzyme synthesizes liponucleotide from either dCTP or CTP at comparable rates (83) but not from ATP, UTP, and GTP (60). In vivo the level of CDP-diglyceride accounts for 60-80% of the total liponucleotide, the rest occurs as dCDP-diglyceride (59,85). The levels of liponucleotide in vivo are very low partly because CDP-diglyceride is quickly converted to other phosphoglycerides and also because there is in *E. coli* a very active CDP-diglyceride hydrolase which converts liponucleotide to phosphatidic acid and CMP (84,86). This enzyme, however, has no affinity for dCDP-diglyceride and one could therefore suggest that it is implicated in the regulation of the CDP-diglyceride to dCDP-diglyceride ratio (84,86).

The formation of CDP-diglyceride leads to a branch point in phosphoglyceride biosynthesis (cf Scheme 2). The phosphatidyl moiety can be transferred to the hydroxyl group of L-serine or to that at the 1-position of *sn*-glycero-3-phosphate (61,62). Since the levels of phosphoglyceride classes occur in definite proportion some mode of regulation might well be exercised at the branch point to account for this phenomenon. Such a regulation mechanism, if indeed it exists, is clearly not understood at the moment.

(d) Synthesis of Phosphatidylserine

CDP-diglyceride:L-serine *O* - phosphatidyltransferase (phosphatidylserine synthetase) forms phosphatidylserine from CDP-diglyceride and L-serine (62).

The coliform enzyme is not bound to the inner membrane in significant amounts but is recovered in association with ribosome-fractions after homogenization (58). The physiological significance of this finding is not certain. Cronan has suggested that it may be an artifact (47). The enzyme can be extracted from ribosomes with 5 M NaCl and has been purified from such extracts some 5,000-fold to homogeneity (87,88). In purified form, it catalyzes the transfer of 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). It catalyzes the reversal of phosphatidylserine synthesis and the slow hydrolyses of CDP-diglyceride and phosphatidylserine to yield phosphatidic acid (87,88). This enzyme, which does not require added cations for activity, is greatly stimulated by Triton X-100 (62,87,88). It has been proposed that the enzyme acts on mixed micelles of Triton X-100 and CDP-diglyceride (87) but a direct effect of the detergent on the enzyme has not been precluded. The Km for serine has been determined to be 0.1 to 1 mM depending on the conditions, and added lipids do affect this kinetic parameter (62,87-89). CDP-diglyceride and dCDP-diglyceride are both active as substrates and

are well preferred to the other liponucleotides tried, ADP-diglyceride and UDP-diglyceride (87,88).

Present evidence, based on genetic and biochemical data, indicates that the major pathway for phosphatidylserine synthesis involves the phosphatidylserine synthetase enzyme (62,90-92). In eucaryotic organisms phosphatidylserine is not synthesized by this *de novo* process but depends on an exchange of the base of phosphatidylethanolamine for serine (87).

Mutants defective in phosphatidylserine synthetase have been isolated. The defect in one mutant was an exaggerated thermosensitivity of the enzyme. At nonpermissive temperatures, the synthesis of phosphatidylethanolamine stops and there are large increases in anionic lipids, especially cardiolipin (92,93). However this genetic evidence does not preclude the possibility of alternative pathways for phosphatidylethanolamine synthesis in vivo. In fact there are mutants which have been isolated that can neither synthesize phosphatidylethanolamine nor accumulate phosphatidylserine. These mutants have a normal phosphatidylserine synthetase and their mutations are located on the genetic map at a position distinct from those of phosphatidylserine synthetase mutants (92).

(e) Synthesis of Phosphatidylethanolamine

Phosphatidylserine is very rapidly converted to phosphatidylethanolamine by a membrane bound phosphatidylserine decarboxylase (58,62). Hydroxylamine treatment of wild type cells (58) or growth of phosphatidylserine decarboxylase mutants at

nonpermissive temperatures (95) results in the accumulation of large amounts of phosphatidylserine.

The enzyme has been extracted from the membrane with Triton X-100 and purified 3,600-fold to homogeneity by a combination of ion-exchange chromatography, gel filtration, and density gradient centrifugation (96). The subunit molecular weight is 36,000 but the native molecular weight is not known (96). The structure of its membrane attachment is also not known.

Inhibition of the decarboxylase by hydroxylamine suggests that there is an aldehyde or ketone group at the active site (96,97). Pyruvate has been shown to be the bound cofactor rather than pyridoxal phosphate (97). The substrate specificity has not been thoroughly examined but the enzyme does not act on free serine nor on glycerophosphorylserine (62,96). It has a marked requirement for Triton X-100 but does not depend on any divalent metal ions (96).

In mutants defective in phosphatidylethanolamine synthesis, there is no rapid and severe effect on growth rate. This is in contrast to mutants blocked early in phospholipid synthesis, for example *gps* A and *pls* B mutants, which grow for about half a generation after phospholipid synthesis is halted by the lack of added precursors in the medium (98).

(f) Synthesis of Phosphatidylglycerophosphate

CDP-diglyceride:*sn*-glycero-3-phosphate phosphatidyltransferase (phosphatidylglycerophosphate synthetase) catalyzes the formation of phosphatidylglycerophosphate from CDP- (or dCDP-) diglyceride and *sn*-glycero-3-phosphate (61,99). This synthetase has been extracted

from *E. coli* and purified 6,000-fold to homogeneity by affinity chromatography on CDP-diglyceride-modified Sepharose (99,100). The apparent subunit molecular weight is 24,000, as judged by polyacrylamide gel electrophoresis (99). Triton X-100 and Mg^{++} or Mn^{++} stimulate the enzyme activity (61,99).

The mechanism of phosphatidylglycerophosphate synthetase may differ from that of phosphatidylserine synthetase since exchange reactions between CMP and CDP-diglyceride or between *sn*-glycero-3-phosphate and phosphatidylglycerophosphate do not occur (61,88,99). By incubating phosphatidylglycerophosphate with CMP, independent investigators have been able to demonstrate a reverse reaction (61,99). Phosphatidylglycerophosphate synthetase is very active only with CDP-diglyceride or its deoxy analogue. The dCDP-diglyceride is about twice as effective as CDP-diglyceride at high concentrations (101).

(g) Synthesis of Phosphatidylglycerol

Phosphatidylglycerophosphate is rapidly dephosphorylated to phosphatidylglycerol (102) by the action of a specific particulate phosphatase which requires Mg^{++} and Triton X-100 for activity (102). The enzyme has been purified only 10-fold but it does not act on *sn*-glycero-3-phosphate or on phosphatidic acid (102). The very marked activity of this enzyme in vivo could explain why phosphatidylglycerophosphate does not accumulate in this organism.

Some formation of phosphatidylglycerol has been obtained in crude extracts of *E. coli* which apparently cannot be attributed

to the combined phosphatidylglycerophosphate synthetase and phosphatase activities (103). This phosphatidylglycerol could arise by the reversal of cardiolipin synthesis (104) or hydrolysis of this polyglycerophosphatide (105-107).

(h) Synthesis of Cardiolipin

Cardiolipin synthase is a membrane bound enzyme which has been found in *E. coli* as well as other bacteria (104). It catalyzes a transphosphatidylation reaction between 2 molecules of phosphatidylglycerol to yield cardiolipin and glycerol as products.

Stanacev *et al* (108) had proposed that cardiolipin is formed by a reaction catalyzing phosphatidylglycerol and CDP-diglyceride as is the case in mammalian mitochondria (109). However the transphosphatidylation reaction appears to be the only pathway involved; albeit, CDP-diglyceride does stimulate the reaction 3-fold (104,108). Accordingly, the phosphatidyl moiety of [^{14}C]-labelled CDP-diglyceride does not incorporate into cardiolipin in vitro in *S. aureus* or *E. coli* extracts (104,110).

D. The Turnover of Phosphoglycerides in *E. coli*

While phosphatidylethanolamine pools in normally growing *E. coli* cells remain stable, those of other phospholipids undergo quite noticeable turnover. Phosphatidic acid, CDP-diglyceride, phosphatidylglycerophosphate, and phosphatidylserine are metabolized very rapidly as intermediates in the biosynthesis of the major phosphoglycerides (44,60-62,102).

The polyglycerophosphatides, phosphatidylglycerol and cardiolipin also undergo turnover but at moderate rates (106,111). The turnover of phosphatidylglycerol could be partly explained on the basis of its conversion to cardiolipin or to acyl phosphatidylglycerol (52,112,113). That of cardiolipin is accountable at least in part by the action of a cardiolipin phosphodiesterase detected in *E. coli* extracts and which has been shown to be active in intact cells (105-107). Levels of cardiolipin and phosphatidylglycerol can be changed, one at the expense of the other by varying the energy supply in the culture medium (35,106,114). Presumably, this interplay calls upon the activity of the polyglycerophosphatide cycle, comprising the cardiolipin synthase and phosphodiesterase enzymes, which appears to be controlled by the levels of energy supply to the cell (106,107).

The turnover of polyglycerophosphatides has also been implicated in the formation of a family of oligosaccharides containing glucose, *sn*-glycero-1-phosphate, succinic acid, and phosphorylethanolamine and termed membrane-derived oligosaccharides (MDO) (55). The unusual stereochemistry and the time course of labelling of these oligosaccharides suggest that the *sn*-glycero-1-phosphate moiety is derived from the polar headgroups of the polyglycerophosphatides (55). Mutants unable to snythesize the membrane-derived oligosaccharides show an important reduction in the rate of polyglycerophosphatide turnover; therefore, much of the turnover must be accountable by MDO synthesis. *sn*-1,2-Diacylglycerol is generated as a by-product of the glycerophosphate transfer to MDO. This diglyceride could then be phosphorylated to phosphatidic acid by the diglyceride kinase.

Although phosphatidylethanolamine is stable under normal conditions, unusual accumulation or turnover of this lipid has been shown. Cells that are treated with phenylethanol showed an apparent conversion of phosphatidylglycerol and cardiolipin to phosphatidylethanolamine (115). *E. coli* B_{FAD} which shows an abnormal envelope metabolism by shedding phosphoglyceride, lipopolysaccharide, and phospholipase A to the medium during growth also displays considerable phosphatidylethanolamine turnover (116). A marked turnover of phosphatidylethanolamine was noticed in a temperature-sensitive DNA initiation mutant of *E. coli* at the restrictive temperature of 40°C (117). A marked turnover of this lipid is also noticed in amino acid starved *E. coli* cells which had a lesion in RNA control (118).

E. Phospholipid Breakdown in *E. coli*

Several types of phospholipase A_1 have been reported to occur in *E. coli*. Fung and Proulx (119,120) first observed that *E. coli* possessed phospholipase A_1 activity and some phospholipase A_2 activity whereas Okuyama and Nojima concurrently reported phospholipase A activity without specifying positional specificity (121). Scandella and Kornberg (122) purified a phospholipase A_1 from the particulate fraction to near homogeneity. The purified enzyme required Ca^{++}, displayed an optimum pH of 8.4 and hydrolyzed only the 1-acyl ester of phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin at comparable rates under the conditions tested. It also displayed considerable lysophospholipase activity. A less active acid phospholipase A_1 has been detected in whole cell homogenates (120) and in *E. coli* spheroplasts (123) but has not been purified. Doi *et al* (124) have designated two kinds of phospholipase A in *E. coli*, detergent-resistant and a detergent-sensitive phospholipase A. Further work on the positional specificity of the detergent-resistant phospholipase A showed that this enzyme had both A_1 and A_2 activities (125). Albright *et al* (126) found both A_1 and A_2 activities in the cell wall but could neither establish nor preclude that these involved two different enzymes. The other detergent-sensitive phospholipase A is located in the cytoplasm and acts mainly on phosphatidylglycerol (124). The enzyme has not been purified and its positional specificity is unknown.

Three types of lysophospholipase activity have been detected in *E. coli* (126). There is a lysophospholipase A_1 activity in the cell wall that seems to be associated with phospholipase A_1. There is also a lysophospholipase A_1 in the membrane and cytosol and a lysophospholipase A_2 in the inner membrane.

The cytosol lysophospholipase has been purified to near homogeneity (127). This enzyme hydrolyzes the 1-acyl and 2-acyl derivatives of glycerophosphorylethanolamine, 1-acylglycerophosphorylglycerol, and 1-acylglycerol, but does not attack diacyl phospholipids.

Lipase activity has also been detected in *E. coli* (128). The enzyme involved hydrolyzes triglycerides, diglycerides, monoglycerides, and simple fatty acid esters at an alkaline pH and requires Ca^{++} together with detergents for activity. In crude homogenates, the level of lipase activity towards trioleoylglycerol is similar to the level of phospholipase A_1 activity (122). Doi and Nojima (129) have confirmed these results and gave genetic evidence indicating that a single enzyme is associated with triglyceride and phospholipid hydrolyses.

Proulx *et al* (130) have purified an enzyme from *E. coli* some 500-fold which displayed predominantly phospholipase A_1 activity but which copurified with lipase, phospholipase A_2, and lysophospholipase. All four activities showed similar susceptibility to heat treatment. The phospholipase A and lipase activities were recovered in a single band when subjected to SDS gel electrophoresis. They all required Ca^{++} for activity. Phosphatidylcholine, phosphatidylethanolamine, and their lyso analogues were all hydrolyzed at equivalent rates which were greater

than the rates of methylpalmitate or triglyceride hydrolyses. Recently, the work of Nojima *et al* (131) has shown that a single enzyme accounts for at least three lipolytic activities, phospholipase A_1, phospholipase A_2, and lysophospholipase activities. *E. coli*, therefore, appears to possess a lipolytic enzyme of broad substrate specificity acting mainly at position 1 but also at position 2 of phosphoglycerides and perhaps also on triglycerides and methyl fatty acid esters.

Mutants lacking this phospholipase activity of broad specificity have no obvious defects in growth and the turnover of their polyglycerophosphatides occurs normally (132-134). Phosphatidylethanolamine is a stable pool in normal and in mutant strains lacking this enzyme (111,121, 133,134). The function of this enzyme is probably related to absorption of lipids from the medium. It is not related to the overall or partial turnover of endogenous phosphoglycerides in normal cells (111,116).

Phospholipase C has been reported to occur in a number of bacterial strains (121,135-137); however, it was not detected in several *E. coli* strains examined by Bright-Gaertner and Proulx (111) and its involvement in the turnover of phosphoglycerides of normally growing and cold shocked *E. coli* cells could not be demonstrated (111).

Phosphatidic acid phosphatase activity has been detected in crude *E. coli* extracts. The enzyme which requires no cations for its activity is most active against lysophosphatidic acid, although significant activity is seen with the diacyl analogue (76). The importance of this enzyme in the metabolism of phosphoglycerides of *E. coli* is unknown since

according to published evidence, diglyceride is not a precursor of the diacylglycerol moiety of phosphoglycerides.

A cardiolipin-specific phosphodiesterase has been found in *E. coli* and other Gram-negative bacteria, catalyzing a reaction similar to that of phospholipase D (105-107,138). The enzyme which hydrolyses cardiolipin to yield phosphatidylglycerol and phosphatidic acid has a requirement for Mg^{++}, is stimulated by ATP and inhibited by detergents and EDTA (107,138).

Extracts of *E. coli* also contain a phosphodiesterase which cleaves glycerophosphorylethanolamine to yield *sn*-glycero-3-phosphate and ethanolamine (126). Thus a complete breakdown of phosphatidylethanolamine may be performed in *E. coli* (126).

There is also an enzyme in *E. coli* which splits CDP-diglyceride to CMP and phosphatidic acid (84,86). This enzyme is cytosolic and appears to be specific for liponucleotides as the deoxyriboliponucleotide analogue is not cleaved (84). On this basis a regulatory function has been proposed for this enzyme since the ratio of ribo- to deoxyriboliponucleotides may have some significance in the regulation of phospholipid composition.

F. The Regulation of the Synthesis of Phosphoglycerides

Factors regulating phospholipid synthesis are not yet clearly understood. Phospholipid and fatty acid formation are usually tightly linked in *E. coli* (35,139). Starving of glycerol autotrophs causes cessation of both phospholipid and fatty acid synthesis (140). The synthesis of lipids is usually coupled to that of other molecules such

that if cells are repressed for a required amino acid, not only protein and RNA synthesis stops but that of phosphoglycerides also (118). Likewise, when temperature-sensitive glycerophosphate acytransferase mutants were subject to a restrictive temperature, not only phospholipid formation stopped, but DNA, RNA, and protein syntheses also (118).

Both glycerophosphate acyltransferase and phosphatidylglycerol-phosphate synthetase are inhibited by high concentrations of guanosine 5'-diphosphate-3'-diphosphate (141). The first enzyme is blocked only when acyl-CoA is the acylating agent (61,142). Since in vitro inhibition by ppGpp is irreversible and in vivo inhibitors of phospholipid synthesis are reversible (121,143), it is not likely that ppGpp is indeed a regulatory substance.

The control of normal headgroup ratios in membrane phospholipids is still unknown. The CDP-diglyceride branch point presumably plays a role. The sum of phosphatidylserine plus phosphatidylethanolamine divided by the sum of phosphatidylglycerol plus cardiolipin is always the same in normal growing cells (95).

Several hypotheses have been proposed for the regulation of headgroup ratio. Kito *et al* (144) have observed separate pools of phosphatidic acid in some membrane preparations. This was based on two facts: (1) phosphatidylethanolamine and phosphatidylglycerol had different fatty acid compositions (145), and (2) endogenous membrane bound phosphatidic acid incubated with sufficient phosphatidylserine synthetase and serine produced phosphatidylethanolamine at a maximum rate. At this point addition of phosphatidylglycerophosphate synthetase

in the presence of α-glycerophosphate caused phosphatidylglycerol to be formed (144). Their conclusions however are at variance with other workers who indicate that only one pool of phosphatidic acid and CDP-diglyceride exists (146-148). Both phosphatidyltransferases forming phosphatidylserine and phosphatidylglycerophosphate use CDP- and dCDP-diglyceride effectively (87,99) and thus the preferential use of CDP-diglyceride for one branch and dCDP-diglyceride for the other is unlikely. Phosphatidylserine synthetase does not limit the levels of phosphatidylethanolamine formed since a 10-fold overproduction of this enzyme in certain mutants does not significantly alter the polar headgroup composition (148). The ratio of neutral to negatively-charged phosphatides present in the membrane may regulate the activity of the phosphatidyltransferases since it was found that various lipids influence the rate of phosphatidylserine synthetase in vitro (89). Because phosphatidylserine synthetase is not apparently membrane-bound, changes in membrane structure may limit the access of the membrane to its membrane bound substrate, CDP-diglyceride (83). The exploration of the regulation of phospholipid headgroup composition is still in the early stages and remains largely unknown.

AIMS OF RESEARCH

Preliminary experiments with *E. coli* extracts indicated that $[^{32}P]$-phosphatidic acid incorporated its label only in polyglycerophosphatides under conditions which according to published data should have permitted formation of labelled phosphatidylethanolamine (106). The aim of the present research was to study further the factors governing incorporation of labelled phosphatidic acid into more complex lipids of *E. coli* and to detect and elucidate with the use of isotope-labelled precursors any pathways for the synthesis of phosphatidylethanolamine hitherto unpublished. This led to the study of the mechanism whereby diglyceride rather than phosphatidic acid is incorporated into phosphatidylethanolamine.

MATERIALS AND METHODS

A. Materials

Escherichia coli B (ATCC 11303) and *Escherichia coli* B_{FAD} were obtained from the American Type Culture Collection and from Dr. J. Cronan, Harvard School of Medicine, respectively.

E. coli B cells grown commercially in a high peptone medium to the late log stage were purchased as a frozen sediment of cells either from General Biochemicals Inc. or from Grain Processing Corp., Iowa.

Tris (hydroxymethyl) aminomethane (Tris), potassium phosphate, sodium phosphate, and all other common chemicals of the purest grade possible were purchased from Fisher Scientific Co. Nucleotides and the phosphoglycerides, beef heart cardiolipin, phosphatidic acid (sodium salt), phosphatidylethanolamine (egg), phosphatidylserine, and phosphatidylglycerol were obtained from Sigma Chemical Co. and/or from Serdary Research Laboratories Inc. Chloroform, methanol, petroleum ether, ethyl ether, and toluene were purchased from Fisher Scientific Co. or J.T. Baker Co. Chloroform was redistilled prior to use.

Cytidine 5'-diphospho-[2-^{14}C]-ethanolamine and PSC solubilizer were purchased from Amersham/Searle Co. $H_3{}^{32}PO_4$, L-[U-^{14}C]-serine, [U-^{14}C]-glycerol, L- [U-^{14}C]-glycerol 3-phosphate, [1,2-^{14}C]-acetic acid (sodium salt), and [1,2-^{14}C]-ethanolamine were purchased from New England Nuclear Corp.

Bactopeptone and yeast extract were purchased from Difco Co. Silica gel G was obtained from Macherey, Nagel, and Co.

B. Methods

(i) Growth of *E. coli B* cells

Stock cultures of *E. coli B* and B_{FAD} were maintained on agar slants. 5 mL broth cultures prepared from the agar slants were used as innocula for the litre cultures. Cells were grown to the late exponential phase at 37°C in 1 L of medium containing 15 g of Bactopeptone, 1 g yeast extract, 5 g sodium chloride, and 20 g of glucose. The glucose was autoclaved separately. Aeration was maintained by slow agitation of the medium. The purity of the culture was checked by the Gram stain, and by plating on MacConkey and citrate agars. Stock cultures were checked by the IMVIC test. Cells were harvested by centrifugation at 4,000 X g for 10 min in a Sorvall automatic refrigerated centrifuge (model RC2-B) at 4°C.

(ii) Preparation of cell-free extracts of *E. coli B* cells

Fresh *E. coli B* cells prepared in this way were suspended in 12 mL of 0.1 M potassium phosphate buffer pH 7.4. The cell suspension was then subjected to ultrasound treatment during four 1 min periods in an ice bath with a Biosonik II Ultrasonicator (Bronwill Scientific Co.) at a setting of 60 watts or a Rapidis cell disruptor (Ultrasonics Ltd.) at maximum power tuned at 6-7.

Between periods the suspension was allowed to cool so that the temperature never exceeded 10°C. The suspension was then centrifuged at 3,000 X g for 10 min at 4°C to remove unbroken cells. Supernatant and particulate fractions were obtained from the cell-free homogenate by centrifugation at 45,000 X g for 40 min in the Sorvall centrifuge. The particulate fraction was suspended in 10 mL of the same buffer. All preparations of *E. coli* cells were dialyzed against 0.1 M potassium phosphate, pH 7.4, unless otherwise stated. The dialysis was carried out in cellulose dialysis tubing for 22 hours, with several changes of buffer. *E. coli* B_{FAD} preparations were made in the same manner.

E. coli B cells grown commercially were suspended in potassium phosphate buffer and cell-free homogenates were obtained as described for freshly prepared cells.

The protein concentrations of all enzyme preparations were determined by the method of Lowry *et al* (149).

(iii) Extraction of Lipids

Lipids were extracted from various sources by the method of Bligh and Dyer (150). A proportion of 2 volumes of methanol to 1 volume of chloroform to 0.8 volume of the aqueous suspension was maintained. This monophasic mixture was then stirred at room temperature and 1 volume each of chloroform and water were added. The resultant biphasic mixture was again stirred. The mixture was then centrifuged at 600 X g for 2 min and the bottom layer was transferred to an

evaporating flask with a Pasteur pipette. The lipid extract was evaporated to dryness in a rotary evaporator at 50°C.

With large extraction volumes such as substrate preparations, the monophasic system was mixed for 20 min and the biphasic system for 5 min on a mechanical stirrer, while with small extraction volumes such as enzyme assays, both systems were mixed 20 seconds on a vortex mixer.

(iv) Separation of Lipids

(a) Phosphoglycerides

Silica gel G plates, 0.5 mm thick, were made from a slurry containing 45 g of silica gel G in 90 mL of distilled H_2O. In initial studies the plates were activated for 60 min at 110°C prior to use but subsequently better separations of lipids were obtained when the activation time was reduced to 20-30 min. The separation of phosphatidic acid from its lyso analogue or other acidic phospholipids was accomplished with silica gel H plates containing no gypsum binder. The plates were prepared from a slurry containing 25 g of silica gel H in 90 mL of 1% Na_2CO_3.

Phosphoglycerides were separated on these plates with the following solvent systems (45):

System 1 - Chloroform:methanol:water (65:25:4 by vol.)

System 2 - Chloroform:methanol:glacial acetic acid (65:25:8 by vol.)

System 3 - Chloroform:methanol:conc. ammonia: water
(70:30:4:2 by vol.)

System 4 - Chloroform:methanol:7 N ammonia
(60:35:5 by vol.)

For routine analysis of the products of enzyme assays, phospholipids were separated on silica gel G plates developed with system 1. The R_F values are given in Table 1.

(b) Neutral Lipids

Neutral lipids were separated on silica gel G plates with solvent systems 5a [petroleum ether:ethyl ether:formic acid (65:35:1.5 by vol.)] and 5b [petroleum ether:ethyl ether:formic acid (75:25:1.5 by vol.)]. In some assays the plates were run in solvent system 5a or 5b, air dried for 20 min, and subsequently run in system 1. The R_f values are given in Table 2.

(v) Mild Alkaline Hydrolysis of the Phosphoglycerides and the Separation of the Water-Soluble Products

In order to identify the products of enzyme assays and to determine the identity and purity of prepared substrates, the phospholipids were degraded to their water soluble products by mild alkaline hydrolysis (151). The lipid sample was dried under a stream of N_2 and dissolved in 0.5 mL of methanol-chloroform (3:2 by vol.). 0.5 mL of 0.2 N methanolic NaOH was added and after the hydrolysis had proceeded at room temperature for 15 min, the water soluble products were extracted by the addition of 1.0 mL of methanol-

Table 1

R_f Values of Phosphoglycerides in Various Solvent Systems

Phosphoglyceride	Chromatographic System			
	1	2	3	4
PA	0	0.58	0.15	0.08
PG	0.32	0.56	0.50	0.52
PE	0.40	0.35	0.55	0.38
CL	0.68	0.95	0.55	0.55
PS	0	0.17	-	0.15

Table 2

R_f Values of Neutral Lipids in Different Chromatographic Systems

Lipid	System 5	System 5a
Fatty Acid	0.75	0.50
Triglyceride	0.91	0.75
Monoglyceride	0.09	0.03
Diglyceride	0.55	0.19

chloroform (1:4 by vol.) and 0.9 mL water. After mixing briefly the biphasic extract was centrifuged and the upper aqueous phase containing the water-soluble products was removed with a pasteur pipette. This phase was neutralized with 0.3 - 0.5 mL of washed Dowex-50 resin in the presence of 1 drop of 1% phenolphthalein. The resin was removed by centrifugation and the neutralized phase was made slightly alkaline with 1-2 drops of 1.5 M methanolic NH_4OH and evaporated to dryness. The water soluble fraction was then dissolved in methanol:water (10:9 by vol.) and spotted on Whatman No. 1 paper and developed with one of three solvent systems:

System 6 - 90% liquefied phenol:water (140:35 w/w giving a final phenol:water ratio of 5:2 w/w)

System 7 - 1 M ammonium acetate pH 7.5:95% ethanol (35:65 v/v)

System 8 - butanol:acetic acid:water (5:4:1 by vol.)

The chromatograms were run for approximately 17 hours by the ascending technique. The R_f values of these water-soluble hydrolysis products are given in Table 3.

(vi) Detection and Identification of the Phosphoglycerides

The lipid components separated on silica gel plates were routinely revealed by exposure to iodine vapour. The iodine was then allowed to sublimate off the plates in air at room temperature.

Table 3.

R_f Values of the Deacylation Products of Phosphoglycerides

Lipid	Deacylation Product	Chromatographic System		
		6	7	8
PA	α-GP	0.29	0.29	0.23
PG	GPG	0.46	0.63	0.15
PE	GPE	0.63	0.22	0.24
CL	GPGPG	0.15	0.58	0.05
PS	GPS	0.17	-	-

To detect specific phospholipids, a variety of stains was used. Nitrogen-containing phospholipids such as phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine were detected by spraying the plates with a solution of Ponceau red, made by dissolving 2 g uranyl nitrate and 0.05 g Ponceau red in 1 L of 0.01 N HCl. Elution of the stained lipid by Bligh and Dyer extraction removed the dye which partitions entirely into the water-methanol phase.

Phospholipids containing free amino groups such as phosphatidylethanolamine and phosphatidylserine were also detected by spraying the plates with 0.5% ninhydrin in acetone:butanol (1:1). The colour was developed by brief heating at 110°C.

Compounds containing vicinyl hydroxyl groups such as various mild alkaline hydrolysis products and phosphatidylglycerol were identified by the periodate-Schiff reaction (152). Each plate was sprayed with 2% aqueous sodium metaperiodate. After 10 min the plate was placed in a sulphur dioxide atmosphere and then sprayed with Schiff's reagent (1% pararosaniline hydrochloride bleached by saturation with SO_2). The sprayed plate was re-exposed to the sulphur dioxide and the appearance of a dark mauve colour indicated a positive test.

Radioisotope labelled compounds were detected by scanning with an Actigraph III gas flow detector (Nuclear Chicago). Labelled compounds were also identified by autoradiography with Kodak Blue Brand (no screen) X-ray film. Films were developed with Kodak developer and fixer.

(vii) Preparation of $[^{32}P]$-Labelled Phospholipids

(a) *E. coli B* cells were cultured to the stationary phase (10-17 hours) at 37°C in 1 L of basic glucose-peptone broth containing 5 mCi of $H_3{}^{32}PO_4$. Cells were harvested by centrifugation at 4,000 X g for 10 min and the lipids were extracted from the harvested cells by the method of Bligh and Dyer (150) with two chloroform washings of the aqueous phase. The medium was neutralized with 1 N NaOH, reautoclaved, and fresh sterile glucose was added. The medium was then re-innoculated with a 5 mL culture broth of *E. coli* and grown as before. Cells were harvested in the same manner and a second lipid extract was prepared and added to the first.

The total lipid extract was separated by preparative TLC with system 2. Individual phospholipids were eluted from the silica gel by Bligh and Dyer extraction (150) and purified by TLC with system 1. An aliquot of each $[^{32}P]$-phospholipid was subjected to mild alkaline hydrolysis and the water-soluble product was analysed by paper chromatography with system 6. Lipid phosphorous was determined by the method of Bartlett (153).

For the preparation of $[^{32}P]$-phosphatidic acid, the purified $[^{32}P]$-phosphatidylethanolamine was reacted with phospholipase D from cabbage (Sigma Chemical Co.) according to the procedure of Yang *et al* (154) with slight modifications. A chloroform solution of phosphatidylethanolamine was transferred to a 15 mL stoppered test tube and evaporated to

dryness with a N_2 stream. 0.2 mL of 0.4 M acetate buffer pH 5.6 and 0.2 mL of 0.2 M $CaCl_2$ were added along with 20-30 mg of enzyme. The total volume was made up to 1 mL and the reaction was started by adding 0.5 mL of water-washed ethyl ether. The tube was stoppered tightly and the reaction mixture was incubated at room temperature in a shaker for 3-4 hours. At the end of the incubation period, the ether layer was evaporated off and an acidic Bligh and Dyer lipid extraction (150) was performed to recover all of the phosphatidic acid. 1.25 mL chloroform and 2.5 mL methanol were added. After mixing, 1.25 mL 0.2 N HCl and 1.25 mL chloroform were added to form the biphasic system. This was mixed, then centrifuged, and the bottom phase was removed and brought to pH 7.5 with 0.2 N methanolic NH_4OH before evaporating to dryness in a rotary evaporator.

(b) $[^{32}P]$-labelled phospholipids were also prepared from rat liver according to the procedure of Fung and Proulx (119). 3 g of rat liver from a 3 week old rat were rinsed in Krebs-Ringer bicarbonate buffer. The liver was sliced into small pieces and put into a 40 mL centrifuge tube with 2 mL of ice-cold Krebs-Ringer buffer. 1 mCi of $[^{32}P]$ was added and the mixture was incubated for 4 hours at 37°C in a shaking waterbath. Throughout the incubation, a gentle stream of water-saturated 5% CO_2 - 95% O_2 was bubbled through the mixture. After the incubation, the mixture was diluted to 8 mL with H_2O and homogenized with a hand glass

homogenizer. A Bligh and Dyer (150) lipid extraction was performed on the mixture and the phospholipids were separated by TLC as stated above. [^{32}P]-labelled phosphatidic acid was prepared from the [^{32}P]-labelled phosphatidylcholine and phosphatidylethanolamine in the same manner as the *E. coli* [^{32}P]-labelled phosphatidic acid.

(viii) Preparation of [^{14}C]-fatty acid-labelled Phospholipids

E. coli B cells were grown to the stationary phase (17 hours) in 1 L of basic medium containing 250 uCi [1,2-^{14}C]-acetate (sp. act. = 53.5 mCi/mmole). The cells were harvested as previously described and the lipids were extracted by the method of Bligh and Dyer (150). The total lipid extract was spotted on silica gel G plates and the chromatograms were developed first with solvent system 5A. The plates were air-dried and further developed with system 1. The individual phospholipids: phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin were eluted from the plates as usual and each was further purified by chromatography with system 1 or 3. About 1% of the radioactivity was incorporated into the phospholipids. [^{14}C]-phosphatidic acid was prepared from [^{14}C]-phosphatidylethanolamine as described previously. [^{14}C]-1,2 diglyceride was prepared by the action of phospholipase C from *Bacillus cereus* on appropriately labelled phosphatidylethanolamine (155). Crude phospholipase C enzyme was prepared as described by Chu (156).

(1-^{14}C-palmitate)-labelled phospholipids were also prepared from rat liver and 1-[^{14}C]-palmitate (sp. act. = 55.26 mCi/mmole). 2-3 g of rat liver were sliced into 10 mL of 0.02 M Tris-HCl buffer pH 7.2 containing 0.125 M KCl and 0.002 M $MgCl_2$. 10 uCi of [^{14}C]-palmitic acid was dried in a test tube to which 75 mg ATP and 2.5 mg CoA dissolved in 3 mL of the Tris buffer neutralized with 0.1 N NaOH was added. After sonication, 3 mL of liver homogenate was added and incubated for 90 min at 37°C in a shaking waterbath. Lipids were extracted as previously stated and separated by TLC first with system 5B followed by system 1.

(ix) <u>Preparation of [^{14}C]-glycerol-labelled Phospholipids</u>

Method (a):

50 uCi of [^{14}C-(U)]-glycerol (sp. act. = 175 mCi/mmole) were added to a 1 L flask of basic medium along with a 5 mL broth of *E. coli* cells and after 8 hours of growth at 37°C the cells were harvested as previously stated. The lipids were extracted and separated with system 1. [^{14}C]-glycerol-labelled phosphatidylethanolamine was treated with phospholipase D and the [^{14}C]-labelled phosphatidic acid was extracted and purified by TLC with system 1. [^{14}C]-diglyceride was prepared by treatment of [^{14}C]-phosphatidylethanolamine with phospholipase C. The [^{14}C]-diglyceride was purified with the use of system 5.

Method (b):

Rat liver homogenate was used as the enzyme source for the incorporation of L-[U-^{14}C]-glycerol-3-phosphate into phospholipids. 2-3 g of rat liver liver were homogenized in 10 mL of the following solution: 0.2MTris, 0.002 M $MgCl_2$, and

0.125 M KCl brought to pH 7.4 with 1 N HCl. 75 mg ATP and 2.5 mg CoA were dissolved in 5 mL of this buffer and to this solution adjusted to pH 7.4 with 1 N HCl was added 20-50 uCi of L-[U-^{14}C]-glycerol-3-phosphate and 3 mL of the rat liver homogenate. The mixture was incubated for 1 hour at 37°C. The lipids were extracted by the method of Bligh and Dyer (150) and separated by preparative TLC as described previously. [^{14}C]-phosphatidylcholine and [^{14}C]-phosphatidylethanolamine were then used as substrates for the preparation of [^{14}C-] glycerol -labelled diglyceride as described previously except that 250 units of commercial phospholipase C from *Bacillus cereus* (Sigma Chemical Co.) were used. This method gave diglyceride of very high specific activity.

(x) <u>Preparation of Phosphatidylethanolamine from [^{14}C]-Serine</u>

In order to make phosphatidylethanolamine in which mainly the ethanolamine group was labelled, *E. coli B* cells were grown with 50 uCi L-[^{14}C-(U)]-serine (sp. act. = 153 mCi/mmole) in the glycerol medium described by Raetz and Kennedy (10). The procedure for the growth of the cells and extraction of lipids were the same as that described on pages 33 and 34.

(xi) <u>Preparation of [^{14}C]-Lysophosphatidylethanolamine and [^{14}C]-Lysophosphatidylglycerol</u>

To prepare [^{14}C]-lysophosphoglycerides, the diacyl analogues labelled with ^{14}C in the acyl groups were degraded with

Phospholipase A_2 from snake venom of Crotalus adamanteus as described by Hildebrand and Law (157) with slight modifications by White and Tucker (158). The phospholipid was dried with a N_2 stream in a tube fitted with a stopper and then dissolved in 20 mL of petroleum ether. 30 ul of 1.5 M NH_4OH, 15 ul of SDS and 30 ul of 0.1 M Tris buffer pH 7.2 containing 0.01 M $CaCl_2$ were added along with 3-5 mg of lyophilized venom from Crotalus adamanteus. The addition of SDS stopped any lysophospholipase activity in the venom preparation. The tube was stoppered and shaken at R.T. for 3-4 hours; then the ether was evaporated off with a stream of N_2 and a small amount (2-3 mL) of $CHCl_3$:MeOH (2:1) was added. The solution was spotted on preparative TLC plates and run in system 1. The lyso compounds were detected with iodine vapours and extracted from the silica gel by Bligh and Dyer extraction. If any unhydrolyzed phospholipid remained, it was eluted and subjected to another incubation with snake venom.

(xii) Preparation of Labelled *E. coli* cell-free Homogenate, Supernatant, and Particulate Fractions

E. coli cell-free homogenate, supernatant, and particulate fractions were used as both substrate and enzyme sources. These fractions were labelled in vivo by growth in media containing [^{14}C]-acetate, [^{14}C]-serine, and $H_3{}^{32}PO_4$.

(a) *E. coli* cells were innoculated into 300 mL of the usual glucose-peptone broth medium containing 100 uCi of [^{14}C]-acetate. The mixture was shaken on a rotary shaker for 7 hours at 37°C. The labelled cells were harvested by centrifugation at 4,000 X g for 10 min and suspended in 10 mL of 0.1 M potassium phosphate buffer pH 7.4. The cells were sonicated for four 1 min periods and then centrifuged at 3,000 X g for 10 min to remove unbroken cells. The homogenate was dialyzed against the same buffer for 22 hours with three changes of buffer.

(b) [^{14}C]-serine-labelled cell-free homogenate was prepared in the same manner as the [^{14}C]-acetate-labelled homogenate with 50 uCi of L-[^{14}C(U)]-serine (sp. act. = 150 mCi/mmole) added to the culture medium. Cells were harvested as given in (a).

To prepare the [^{14}C]-serine-labelled supernatant and particulate fractions, the [^{14}C]-serine labelled homogenate was centrifuged at 200,000 X g for 2 hours in a Beckman L2-65B ultracentrifuge. The supernatant was removed and the pellet was suspended in the phosphate buffer. All fractions were frozen and stored at -20°C if not used immediately.

(c) [^{32}P] and [^{14}C]-serine-double-labelled supernatant and particulate fractions were prepared in the same manner as the [^{14}C]-serine labelled fractions with 5 mCi of $H_3{}^{32}PO_4$ and 100 uCi of L-[^{14}C(U)]-serine (sp. act. = 150 mCi/mmole) added to the

300 mL culture medium. Cells were cultured and harvested according to the procedure in (a).

(xiii) Gel Filtration of Labelled cell-free Supernatants

The [^{14}C]-serine and [^{32}P/^{14}C]-serine labelled 200,000 X g supernatants prepared in section (xii) (b) and (c) were analyzed in columns of Sephadex G-50 (fine) and G-150. The columns were 2.5 cm in diameter and 28 cm in height. 0.1 M ammonium bicarbonate buffer pH 7.0 was used as a volatile buffer to suspend the Sephadex and as eluant. 5 mL fractions (flow rate = 0.5 mL per min) were collected and an aliquot was counted in PCS:toluene (2:1) for assessment of radioactivity and also read at 280 nm in a Hitachi Perkin-Elmer double beam spectrophotometer to assess protein. The peaks which contained both radioactivity and protein were lyophilized in a freeze dryer (New Brunswick Scientific Co. Ltd.) and resuspended in 0.1 M potassium phosphate buffer pH 7.4. These protein fractions were stored in a frozen state if not used immediately.

(xiv) Trypsin Treatment of [^{14}C/ ^{32}P] Protein Fractions

The effect of trypsin on the activity of the [^{14}C/^{32}P]-labelled protein fractions prepared as described in the preceding section was tested. The fractions were treated with bovine pancreas trypsin Type VIII (Sigma Chemical Co.) according to the procedure of Doi and Nojima (125). 1 mL of the [^{14}C/^{32}P]-labelled

protein fraction in buffer was reacted with 0.15 mL of trypsin solution (1 mg/mL in 20 mM acetate buffer 3.7) and 0.35 mL of 0.2 M Tris-HCl buffer pH 7.9 for one hour at 37°C in a shaking incubator. To stop the trypsin digestion, 0.02 mL of a solution of soybean trypsin inhibitor (Worthington Biochem. Co.) (5 mg/mL in 20 mM acetate buffer pH 3.7) was added. This trypsin-treated fraction was then used as substrate in the in vitro assays.

(xv) Preparation of R Lipopolysaccharides from *E. coli B*

R Lipopolysaccharides were obtained from commercial *E. coli B* cells, obtained from the Grain Processing Corp., according to the procedure of C. Galanos *et al* (159). The commercial cells were lyophilized in a freeze-dryer for approximately 5 hours. 12.8 g of dry cells were transferred to a centrifuge tube and 50 mL of the extraction mixture - liquid phenol (90%):chloroform: petroleum ether (2:5:8 by vol.) were added. The mixture was then stirred until a fine suspension was obtained. Because the suspension was too viscous, 20 mL more extraction mixture was added. The mixture was centrifuged at 3,000 rpm on an IEC International Centrifuge size 2 model V for 5 min. The supernatant containing the lipopolysaccharide was filtered through filter paper into a round bottom flask. The bacterial residue was washed twice more with the same volume of extraction mixture and the supernatants added to the first extract. Petroleum ether and $CHCl_3$ were removed by rotary evaporation at 40°C. The solution was then transferred

to a glass centrifuge bottle and water was added dropwise until the lipopolysaccharide precipitated. Addition of water was stopped when the lipopolysaccharide started settling down after the mixture was allowed to stand for 1-2 min and no more precipitate formed. The precipitated lipopolysaccharide was then centrifuged at 3,000 rpm for 10 min. The supernatant was decanted and the tube was allowed to stand for 2-3 min upside down. It was then wiped inside with filter paper. The precipitate was washed three times with small portions of 80% phenol (5 mL each) and the inside of the tube was wiped with filter paper after decantation of the supernatant. Finally the precipitate was washed three times with ether to remove any remaining phenol and dried in vacuo. The lipopolysaccharide was taken up in 14 mL of H_2O, warmed to 45^oC and vacuum was carefully applied to remove the air. The lipopolysaccharide was then shaken for a few minutes whereby a viscous solution was obtained. The lipopolysaccharide was centrifuged once at 100,000 X g for 4 hours. The resulting sediment was redissolved in 5 mL of H_2O and transferred to a 40 mL glass centrifuge tube and lyophilized in a larger vacuum bottle. The resulting powder was very hygroscopic. The yield of lipopolysaccharide was 0.38 g.

(xvi) Liquid Scintillation Counting

Liquid scintillation counting was performed in disposable polyethylene vials with a Beckman LS 133 counter. Radioactive areas

on TLC plates which corresponded to known lipids to be analysed were counted without prior elution in the following scintillation fluid: 5 g of 2,5-diphenyloxazole in 1 L toluene mixed with 100 mL methanol and 3.5 mL glacial acetic acid (160). Material from paper chromatograms was counted by cutting appropriate areas into sufficiently large sections and standing these sections upward in the vial. Quench corrections for $[^{14}C]$ counting were estimated by a channels ratio method, those for $[^{32}P]$ counting by an external standard method and when both isotopes were counted together the contribution of $[^{32}P]$ counts in the $[^{14}C]$ channel as well as the quenching were estimated by internal standards. The quenching being relatively constant in our samples, the contribution of $[^{32}P]$ counts in the $[^{14}C]$ channel varied between 20-25% only.

(xvii) Dialysis

All *E. coli* cell-free homogenate, supernatant, and membrane fractions were dialysed unless otherwise stated. Seamless cellulose dialysis tubing (average diameter of 23 mm) was prepared for use by the method described by McPhie (161). 10-15 mL aliquots of *E. coli* homogenate, supernatant, or particulate fractions were dialysed against three changes of 2 L of 0.1 M potassium phosphate buffer pH 7.4 at $4^{0}C$ for 22 hours.

RESULTS

A. The Metabolism of Exogenous Phosphatidic Acid in *E. coli* cell-free Extracts

(i) Hydrolysis of Phosphatidic Acid

This section deals primarily with factors influencing the conversion of phosphatidic acid to other phospholipids. Preliminary experiments indicated, however, that some hydrolysis of [^{32}P]-phosphatidic acid occurred under the conditions chosen for such conversions depending on the usual factors of time of incubation and enzyme concentration. This was assessed by an increase of water-soluble counts following incubation with enzyme although attempts to identify the water-soluble products formed were not made. The pathway for phosphatidic acid breakdown was investigated briefly.

Results summarized in Table 4 indicate that [^{14}C-acyl]-phosphatidic acid is degraded to labelled diglyceride and labelled fatty acid under these conditions. When certain cofactors such as CTP, serine, and α-glycerophosphate were omitted, the hydrolysis to fatty acid proceeded unaffected but that to diglyceride increased. The increase in diglyceride can be explained on the basis that with nucleotides absent, the diglyceride formed cannot be reconverted to phosphatidic acid. Fatty acid accumulation was optimum at pH 9.0 (Figure 3) and was not dependent on cation activation since it was greatest in the absence of added cations or in the presence of EDTA (Table 5).

Table 4

The Hydrolysis of [^{14}C]-Phosphatidic Acid
To Labelled Diglyceride and Fatty Acid

Conditions	Counts Recovered in Products	
	DG *	FA *
standard conditions	1062	2105
without nucleotides, serine and α-glycerophosphate	4098	2262

The standard reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 32,000 dpm [^{14}C-acyl]-phosphatidic acid (sp. act. = 267 dpm/nmole), 0.1% Triton-X-100, 2.67 mg *E. coli* cell-free homogenate protein, 2.8 mM ATP, 0.4 mM CTP, 20 mM serine, 10 mM α-glycerophosphate, and 10 mM $MgCl_2$. The reaction was incubated for 30 min at 37°C.

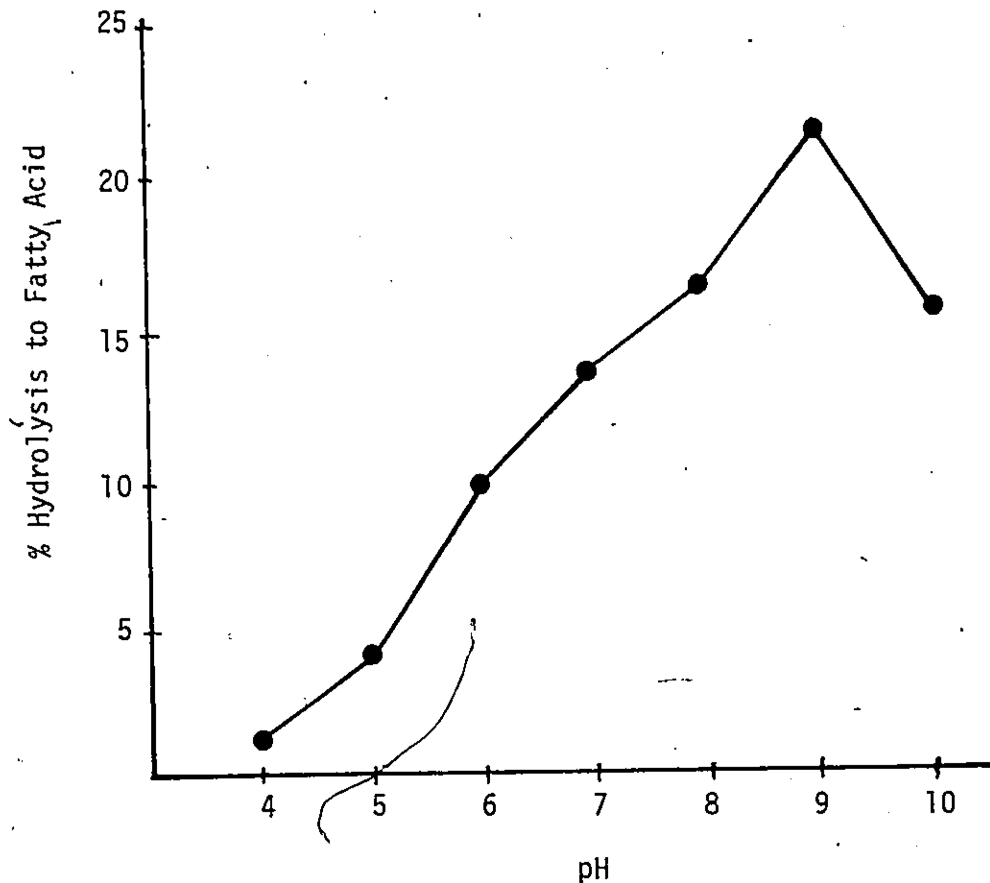
* DG = diglyceride

FA = fatty acid

*The remainder of the counts was accounted for by unhydrolyzed product as well as material in the water phase.

Figure 3

The Effect of pH on the Hydrolysis of
[^{14}C]-Phosphatidic Acid to Labelled Fatty Acid



The standard reaction mixture contained in 2 mL buffer, 45,000 dpm [^{14}C-acyl]-phosphatidic acid (sonicated in buffer, sp. act. = 267 dpm/nmole), 5 mg *E. coli* cell-free homogenate protein. Acetate buffer was used for buffers of pH 4,5, and 6; potassium phosphate buffer for pH 6,7, and 8; and borate buffer for pH 9 and 10. The reaction was incubated for 10 min at 37°C.

Table 5

The Effect of Cations on the Hydrolysis
of [^{14}C]-Phosphatidic Acid to Labelled Fatty Acid

Conditions	Counts Recovered in Products	
	DG	FA
standard conditions	204	3328
+ 5 mM $CaCl_2$	175	303
+ 5 mM $MnCl_2$	173	458
+ 5 mM $MgCl_2$	191	676
+ 5 mM EDTA	298	3464

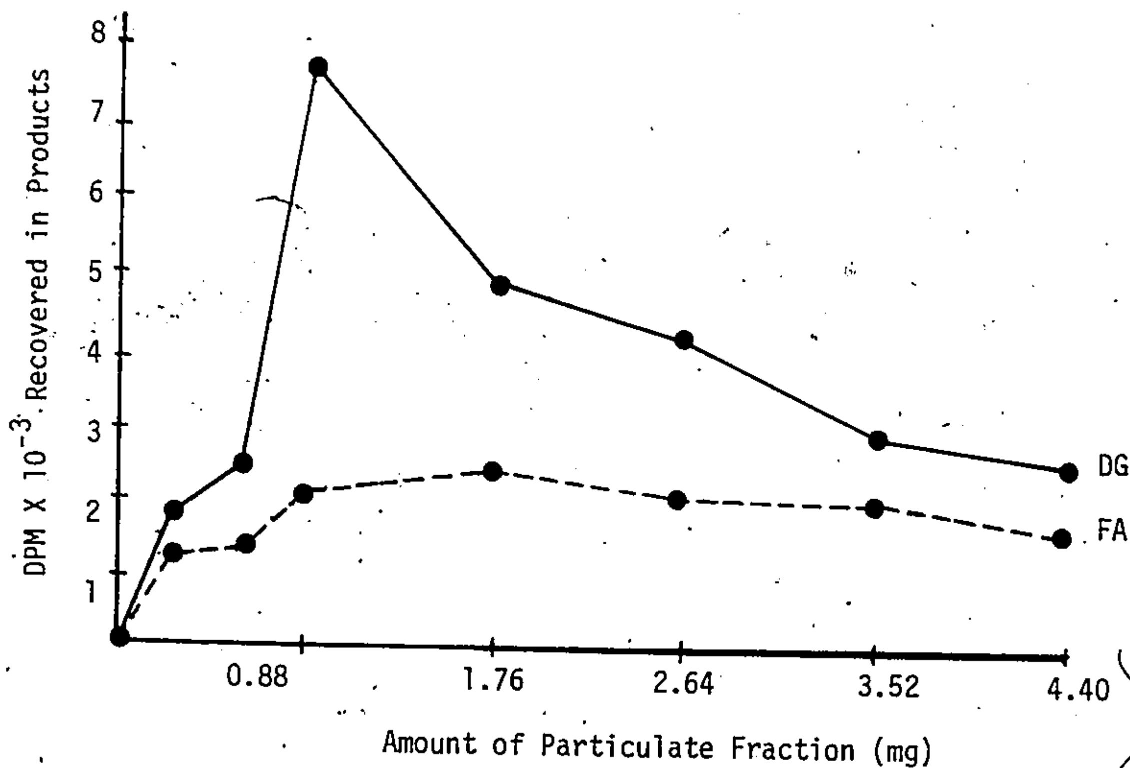
The standard reaction mixture contained in 2 mL 0.1 M Tris-HCl buffer pH 9.0, 25,000 dpm [^{14}C-acyl]-phosphatidic acid (sonicated in buffer, sp. act. = 267 dpm/nmole), 5 mg *E. coli* cell-free homogenate protein, and additives as listed. The reaction was incubated for 10 min at 37°C.

Both labelled diglyceride and fatty acid increased more or less linearly with amount of enzyme up to a concentration of 0.88 mg protein and 1.76 mg protein respectively under the conditions used (Figure 4). At higher concentrations of enzyme the formation of these labelled products decreased. Labelled lysophosphatidic acid formed from [^{32}P] -phosphatidic acid up to a concentration of about 1 mg protein of enzyme and fell at concentrations higher than this (Figure 5). Since crude enzyme preparations were used, binding of phosphatidic acid to non-catalytic proteins could account for the loss of activity at high enzyme concentrations. Alternately, increased conversion of phosphatidic acid to other phosphatides at higher enzyme concentrations would effectively compete with the hydrolytic activity.

On the basis of the results just described, one can explain the hydrolysis of phosphatidic acid by the activity of phosphatidate phosphohydrolase described previously by Van den Bosch and Vagelos (76) and by an acyl esterase acting on phosphatidate. The enzyme should be characterized further before conclusions can be made regarding its mode of action. It is unlikely, however, that the fatty acid liberated is the result of the combined action of phosphatidate hydrolase activity and lipase activity since the latter enzyme requires Ca^{++} for its activation and would be inhibited by EDTA (128). Moreover the formation of [^{32}P] -lysophosphatidic acid from the diacyl analogue necessarily implies an acyl esterase activity. Phospholipase A is probably not implicated here since it also has a strict requirement

Figure 4

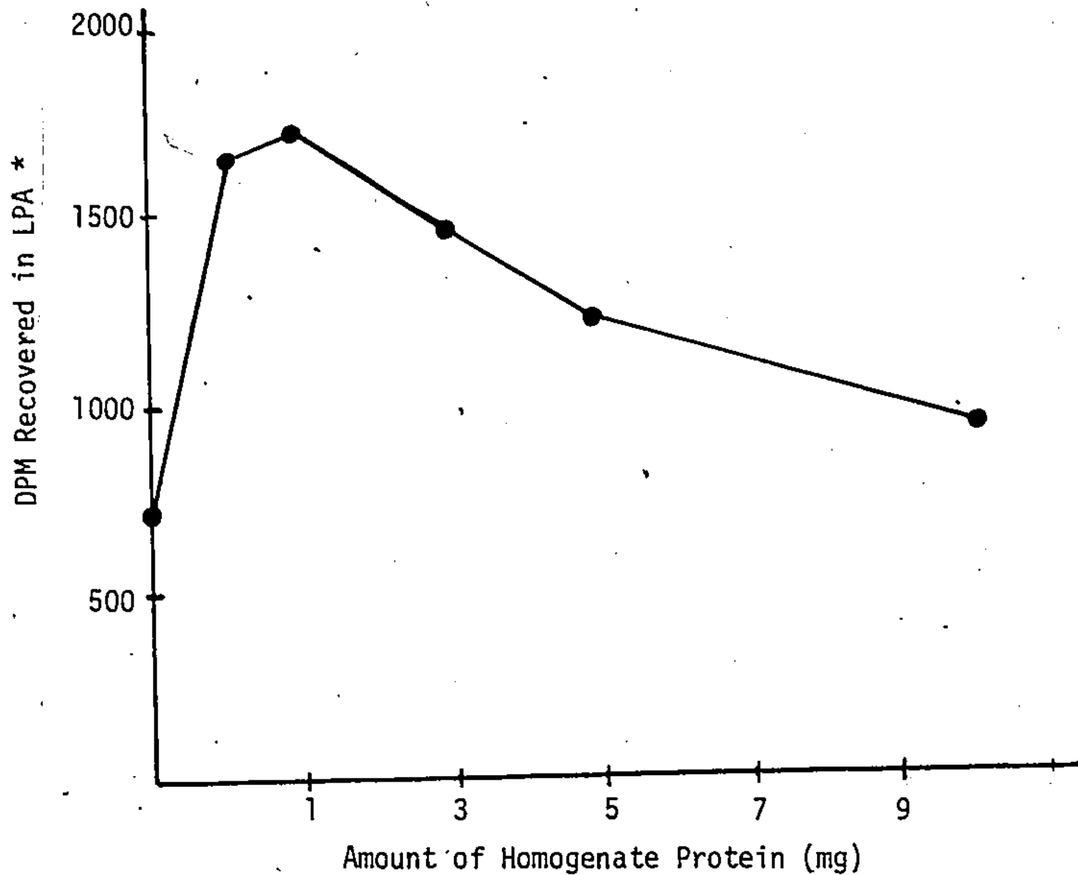
The Effect of Protein Concentration on the Hydrolysis of [14C]-Phosphatidic Acid to Labelled Diglyceride and Fatty Acid



The reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.3, 32,000 dpm [14C-acyl]-phosphatidic acid (sp. act. = 2.67 dpm/nmole), 0.1% Triton X-100, 10 mM $MgCl_2$, and *E. coli* cell-free particulate protein in varying amounts. The reaction was incubated for 30 min at 37°C.

Figure 5

The Effect of Protein Concentration on the Hydrolysis of [^{32}P]-Phosphatidic Acid to Labelled Lysophosphatidic Acid



The standard reaction mixture contained in 2 mL 0.1 M Tris HCl buffer pH 9.0, 14,309 dpm [^{32}P]-phosphatidic acid (sonicated in buffer sp. act. = 3872 dpm/nmole), and *E. coli* cell-free homogenate protein in the amounts indicated. The reaction was incubated for 10 min at 37°C.

* LPA = lysophosphatidic acid

for Ca^{++}. There is likely therefore phosphatidate acyl esterase activity distinct from the other lipolytic activities in *E. coli*.

(ii) <u>The Conversion of [^{32}P]-Labelled Phosphatidic Acid to Other Phosphoglycerides</u>

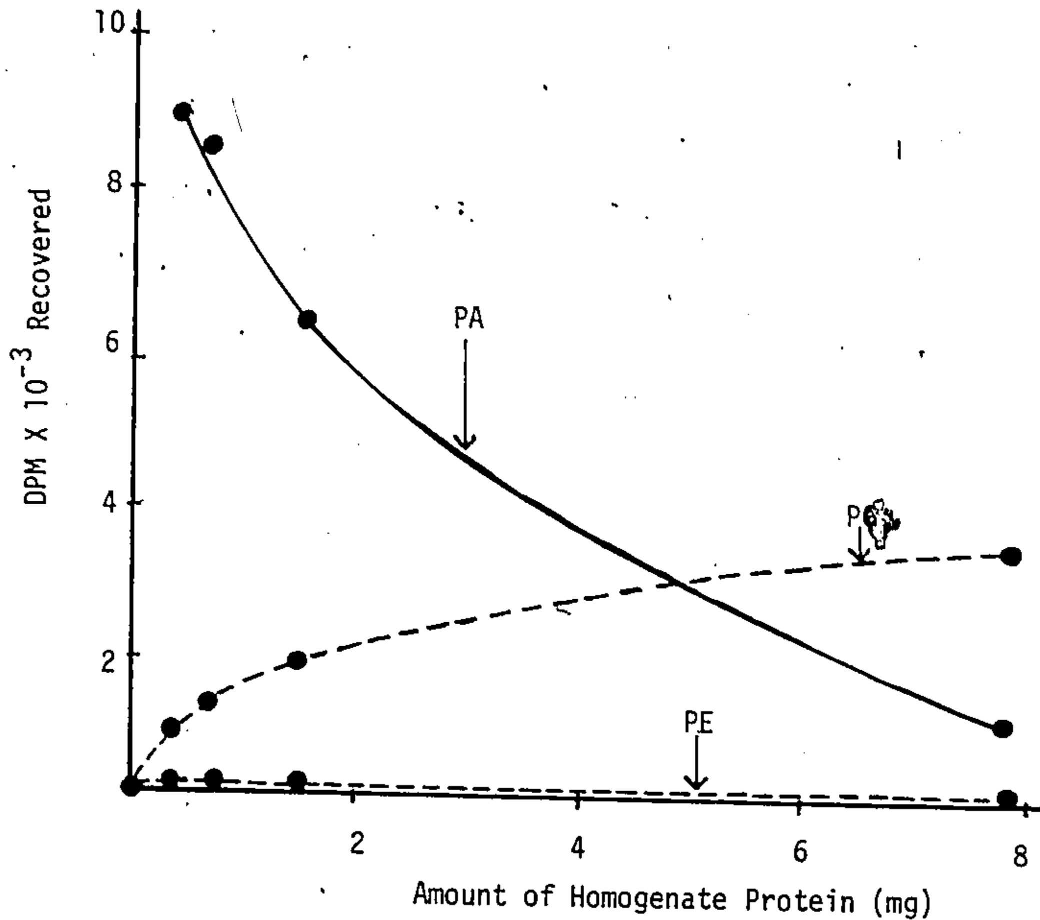
Preliminary work by Audet *et al* (106) indicated that exogenous labelled phosphatidic acid, in the presence of detergent, CTP, and Mg^{++}, was incorporated by cell-free homogenate of *E. coli* into phosphatidylglycerol and cardiolipin but apparently not into phosphatidylethanolamine.

Results shown in Figure 6 indicate the extent of [^{32}P]-phosphatidic acid incorporation into phosphatidylglycerol under conditions which would be expected to be suitable for formation of both phosphatidylethanolamine and the polyglycerophosphatide. These conditions included serine, α-glycerophosphate, Mg^{++}, CTP, and Triton X-100 as additives. The results indicate that with increased protein concentration, the formation of phosphatidylglycerol increased proportionately, but no incorporation occurred in the phosphatidylethanolamine fraction even at higher concentrations of enzyme. Occasionally a small amount of cardiolipin was also formed.

Results summarized in Table 6 show that the formation of [^{32}P]-phosphatidylglycerol is stimulated by CTP, Mg, and α-glycerophosphate. In no case, however, was there formation of phosphatidylethanolamine.

Figure 6

The Effect of Protein Concentration on the Incorporation of [^{32}P]-Phosphatidic Acid into Labelled Phosphatidylglycerol



The reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.3, 11,500 dpm [^{32}P]-phosphatidic acid (sp. act. = 607.1 dpm/nmole), 10 mM $MgCl_2$, 0.4 mM CTP, 20 mM serine, 20 mM α-glycerophosphate, 0.1% Triton X-100, and *E. coli* homogenate protein in varying amounts. The reaction was incubated for 30 min at 37°C.

Table 6

The Effect of Cofactors on the Incorporation of [^{32}P]-Phosphatidic Acid into Labelled Phosphoglycerides

Conditions	Counts Recovered In	
	PG	PE
standard conditions	1489	0
- CTP	302	0
- Mg^{++}	199	0
- α-glycerophosphate	953	0

The standard reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.3, 8.25 mg *E. coli* homogenate protein, 11,500 dpm [^{32}P]-phosphatidic acid (sp. act. = 6071 dpm/nmole), 10 mM $MgCl_2$, 0.4 mM CTP, 20 mM serine, 20 mM α-glycerophosphate, and 0.1% Triton X-100. The reaction was incubated for 30 min at 37°C.

(iii) The Conversion of $[^{32}P]$-Phosphatidic Acid and $[^{14}C]$-Serine to Labelled Phosphoglycerides

Results summarized in Table 7 indicate that when exogenous $[^{32}P]$-phosphatidic acid was incubated together with $[^{14}C]$-serine in the presence of *E. coli* homogenate or particulate preparations, only the $[^{14}C]$-isotope was recovered in the phosphatidylethanolamine fraction under various conditions tested. CDP-diglyceride was added in some cases to show that the phosphatidylserine synthetase and decarboxylase were indeed active in our preparations and especially good incorporation of $[^{14}C]$-serine into phosphatidylethanolamine was obtained under these conditions. However one had to expect a poorer incorporation of $[^{32}P]$-phosphatidic acid into phosphatidylethanolamine in the presence of the liponucleotide because of the isotope dilution effects.

In the absence of CDP-diglyceride, incubations with CTP and Mg^{++} resulted in a lesser but significant incorporation of $[^{14}C]$-serine into phosphatidylethanolamine. There was in this case as well no noteworthy incorporation of $[^{32}P]$ label from phosphatidic acid into phosphatidylethanolamine. The only conversion of labelled phosphatidic acid that occurred involved the formation of polyglycerophosphatides. It is interesting to note that in the present experiments phosphatidylethanolamine synthesis did occur in the particulate fraction. The phosphatidylserine synthetase enzyme was reported to be located mainly in the ribosomal fraction which is sedimented at a much greater gravitational force than the one used

Table 7

The Effect of CDP-Diglyceride on the Incorporation of [^{32}P]-Phosphatidic Acid and [^{14}C]-Serine into Labelled Phosphatidylethanolamine

Conditions	Counts Recovered In Phosphatidylethanolamine	
	^{32}P	^{14}C
with 5.6 mg homogenate protein:		
standard conditions	347	1,291
+ 0.4 mM CDP-diglyceride	255	390,285
with 3.6 mg particulate protein:		
standard conditions	306	17,265
+ 0.4 mM CDP-diglyceride	210	54,261

The standard reaction mixture contained in 1.5 mL 0.1 M potassium phosphate buffer pH 7.3, 78,960 dpm [^{32}P]-phosphatidic acid (sp. act. = 65000 dpm/nmole), 1,110,000 dpm [^{14}C]-serine (sp. act. = 153 mCi/mmole), 10 mM $MgCl_2$, 0.4 mM CTP, 0.1% Triton X-100, and *E. coli* cell-free homogenate or particulate protein as indicated. The reaction was incubated for 30 min at 37°C.

to prepare our own particulate fraction (88). However as Cronan has discussed (47), overly vigorous disruption of the cells can result in a redistribution of the enzyme such that the membrane particulate fraction becomes almost completely deprived of it. Considerable levels of activity are recovered in the lysed envelope fraction if milder disruption procedures are used (87).

One can conclude from the study that exogenous $[^{32}P]$-phosphatidate is not incorporated into phosphatidylethanolamine under conditions which allow formation of polyglycerophophosphatides from this lipid and incorporation of $[^{14}C]$-serine into phosphatidylethanolamine.

(iv) The Effect of CDP-diglyceride on the Conversion of $[^{32}P]$-Phosphatidic Acid to Labelled Phosphoglycerides

Results summarized in Table 8 indicate that exogenous CDP-diglyceride inhibits incorporation of $[^{32}P]$-phosphatidic acid into phosphatidylglycerol. One can suggest from this that the mode of $[^{32}P]$-phosphatidic acid incorporation into polyglycerophosphatides involves its prior conversion to $[^{32}P]$-CDP-diglyceride in accordance with known biosynthesis pathways for phosphatidylglycerol. Addition of unlabelled CDP-diglyceride inhibits labelled polyglycerophosphatide formation because of an isotope dilution effect.

(v) The Conversion of $[^{14}C]$-Phosphatidic Acid to Labelled Phosphoglycerides

Results summarized in Table 9 indicate that $[^{14}C]$-acyl -phosphatidic acid was incorporated into phosphatidylglycerol and also

Table 8

The Effect of CDP-Diglyceride on the Incorporation of [^{32}P]-Phosphatidic Acid into Labelled Phosphoglycerides

Conditions	Counts Recovered in Products		
	PG	PE	CL *
standard conditions	1024	0	594
+ 20 uM CDP-diglyceride	642	0	286
+ 300 uM CDP-diglyceride	284	0	249

The standard reaction mixture contained in 2 mL of 0.1 M potassium phosphate buffer pH 7.3, 8.5 mg *E. coli* cell-free homogenate protein, 10 mM $MgCl_2$, 0.4 mM CTP, 20 mM serine, 20 mM α-glycerophosphate, 0.1% Triton X-100, and 19,700 dpm of [^{32}P]-phosphatidic acid (sp. act. = 6775 dpm/nmole). The reaction was incubated for 30 min at 37°C.

* CL = cardiolipin

Table 9

The Incorporation of $[^{14}C]$-Phosphatidic Acid into Labelled Lipids

Conditions	Net Counts Recovered in Products			
	PG	PE	FA	DG
standard conditions	0	2005	1684	2151
+ 10 mM Mg^{++}	643	129	983	986
+ 10 mM Mg^{++} + 0.4 mM CTP	1272	0	693	1630
+ 20 mM serine	36	1546	1456	1325

The standard reaction mixture contained in 1.6 mL 0.1 M potassium phosphate buffer pH 7.4, 15,280 dpm $[^{14}C\text{-acyl}]$-phosphatidic acid (sp. act. = 227 dpm/nmole), 0.1% Triton X-100, 6.7 mg *E. coli* homogenate protein (sonicated/min), and additives as listed. The reaction was incubated for 30 min at 37°C.

into phosphatidylethanolamine. However, upon Mg^{++} + CTP addition the formation of phosphatidylethanolamine decreases substantially and on one occasion was totally absent. Added serine does not stimulate the incorporation into phosphatidylethanolamine even when dialysed enzyme preparations were used as enzyme. Perhaps endogenous serine is generated during the course of incubation. There was formation of diglyceride and fatty acid under all conditions (approximately 60% diglyceride:40% fatty acid) which also decreased upon addition of Mg^{++} + CTP. As expected the addition of Mg^{++} + CTP stimulates phosphatidylglycerol formation and this makes less phosphatidic acid available for phosphatidylethanolamine synthesis.

Since $[^{32}P]$-phosphatidic acid is not incorporated into phosphatidylethanolamine whereas $[^{14}C]$-phosphatidic acid is, one can suggest that it is the diglyceride moiety of phosphatidic acid that serves to form phosphatidylethanolamine. Exogenous phosphatidic acid is broken down to labelled diglyceride. Addition of nucleotide, which stimulates the reconversion of any diglyceride produced to phosphatidic acid, favours phosphatidylglycerol formation but not that of phosphatidylethanolamine.

B. The Effect of CDP-diglyceride on the Incorporation of $[^{14}C]$-Serine and $[^{14}C]$-*sn*-Glycero-3-phosphate into Phosphoglycerides of *E. coli*

The lack of phosphatidylethanolamine synthesis from $[^{32}P]$-labelled-phosphatidic acid could have been due in part to a much

greater rate of utilization of CDP-diglyceride by the phosphatidylglycerophosphate synthetase than by the phosphatidylserine synthetase. In such a case the very limited supply of the liponucleotide as is known to exist endogenously (59) could result in the almost exclusive synthesis of phosphatidylglycerol.

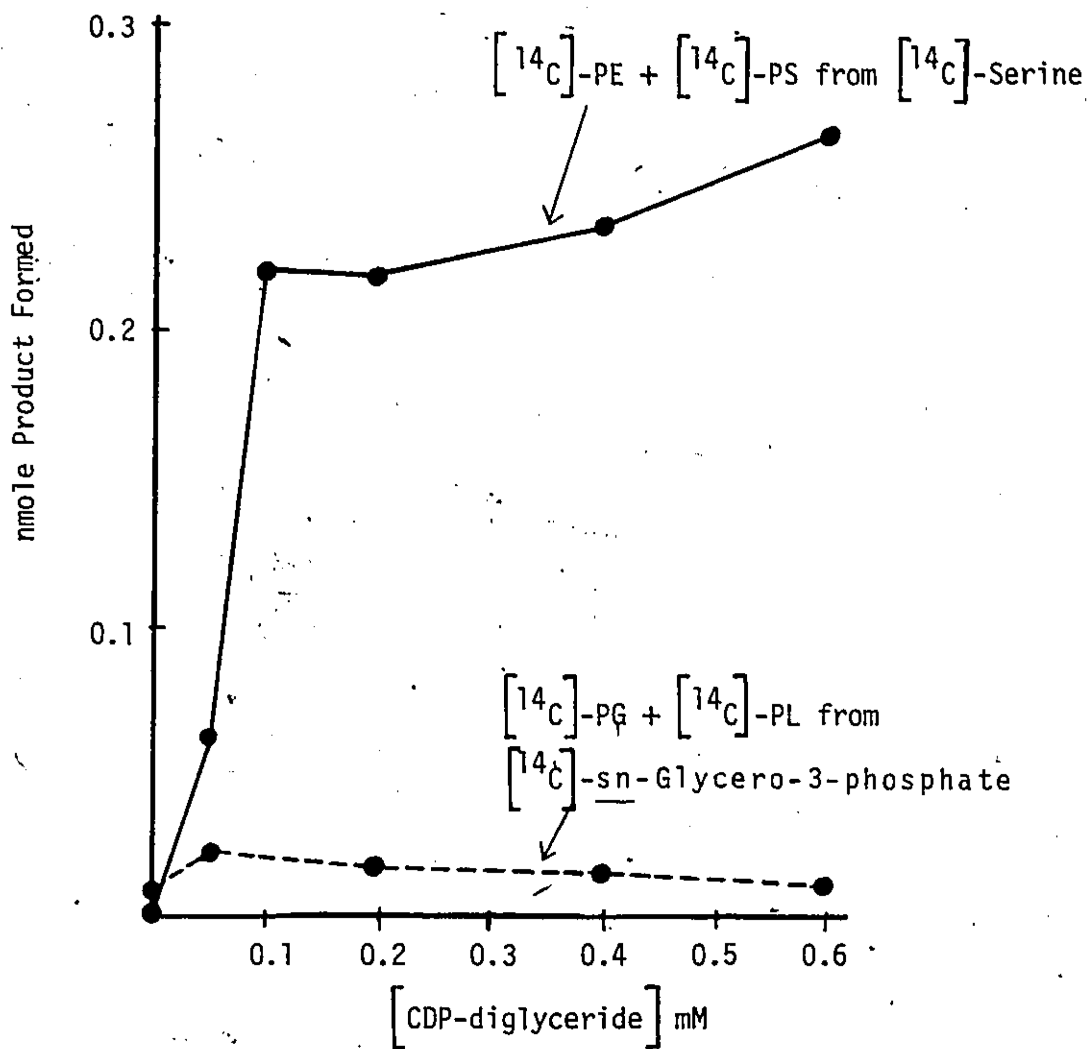
Results illustrated in Figure 7 indicate, however, that for any concentration of CDP-diglyceride added, the synthesis of labelled ethanolamine phosphatide from $[^{14}C]$-serine far exceeded that of labelled phosphatidylglycerol from $[^{14}C]$-*sn*-glycero-3-phosphate when the same amount of labelled precursor was used. This marked difference may not necessarily reflect the relative affinities of the synthetases for the liponucleotide substrates. Differences in the endogenous levels of serine and *sn*-glycero-3-phosphate would result in different isotope dilutions which would in turn reflect on the synthesis of each labelled phosphatide. However, the endogenous glycerophosphate levels have been reported to be very low (76) and homogenate fraction used in this experiment was extensively dialysed. It is therefore unlikely that a more marked dilution of the $[^{14}C]$-α-glycerophosphate occurred unless it was generated in greater amounts than serine during the incubation.

C. The Incorporation of di-$[^{14}C]$-acyl-Glycerol into Phosphoglycerides

The lack of exogenous $[^{32}P]$-phosphatidic acid incorporation into phosphatidylethanolamine under conditions which allowed $[^{14}C]$-

Figure 7

The Effect of CDP-diglyceride on the Incorporation of [^{14}C]-Serine and [^{14}C]-*sn*-Glycero-3-phosphate into Phosphoglycerides



The reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.3, 10 mM $MgCl_2$, 0.1% Triton X-100, 4.8 mg *E. coli* cell-free homogenate protein, 0.51 nmole of either [^{14}C]- sn-glycero-3-phosphate (sp. act. = 392,940 dpm/nmole) or [^{14}C]-serine (sp. act. = 335,220 dpm/nmole), and CDP-diglyceride in the amounts indicated. The reaction was incubated for 30 min at 37°C.

phosphatidic acid incorporation into this lipid prompted an investigation of alternative pathways for the synthesis of this lipid. Since diglyceride readily incorporates into phosphoglycerides of mammalian tissues and since labelled diglyceride did form from $[^{14}C]$-phosphatidic acid under our conditions (Table 9), this neutral lipid was tested as a direct precursor of phosphatidylethanolamine under different conditions.

Results summarized in Table 10 indicate that di-$[^{14}C]$-acyl-glycerol readily incorporates into phosphatidylethanolamine but not as readily into phosphatidylglycerol. A small number of counts was also recovered in a tentatively identified phosphatidylserine fraction. Various cofactor additives were tested to indicate the possible pathway(s) involved in the incorporation into phosphatidylethanolamine but none had a stimulatory effect even when extensively dialysed preparations were used. In fact, addition of cofactors containing serine or ethanolamine base inhibited the incorporation slightly. The enzyme preparation used thus contained or generated some endogenous cofactor which indirectly or directly supplied the phosphorylethanolamine moiety to phosphatidylethanolamine.

Results in Table 11 indicate a direct incorporation of labelled diglyceride into phosphatidylethanolamine since addition of unlabelled fatty acid to the incubation medium had no inhibitory effect on the incorporation. Instead a slight unexplained stimulation was observed. Had the incorporation involved the release of fatty acid from diglyceride prior to incorporation, isotope dilution would have decreased the labelling of phosphatidylethanolamine.

Table 10

The Incorporation of di-[^{14}C]-acyl-Glycerol into Labelled Phosphoglycerides

Conditions	Counts Recovered in Products		
	PS	PG	PE
standard conditions	0	124	3205
+ 20 mM serine	257	124	2401
+ 20 mM ethanolamine	263	92	2597
+ 20 mM phosphoserine	198	81	1797
+ 20 mM phosphoethanolamine	258	96	1928
+ 20 mM CDP-ethanolamine	123	98	2610

The standard reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 2.2 mg *E. coli* particulate protein (dialysed 4 hours), 10 mM $MgCl_2$, 0.4 mM CTP, 0.1% Triton X-100, and 25,000 dpm di-[^{14}C]-acyl-glycerol (sp. act. = 2453 dpm/nmole). The reaction was incubated for 30 min at 37°C.

Table 11

The Effect of Unlabelled Fatty Acid on the Incorporation of di-[^{14}C]-acyl-Glycerol into Phosphatidylethanolamine

Conditions	Counts Recovered in Phosphatidylethanolamine
standard conditions	5312
+ 0.2 mM palmitic acid *	6783
+ 0.5 mM palmitic acid	9192
+ 1.0 mM palmitic acid	8671

The standard reaction mixture contained in 2 mL, 0.1 M potassium phosphate buffer pH 7.4, 4.8 mg *E. coli* homogenate protein, 0.1% Triton X-100, 10 mM $MgCl_2$, 0.4 mM CTP, 20 mM phosphoserine, 20 mM phosphoserine, and 24, 845 dpm di-[^{14}C]-acyl-glycerol (sp. act. = 2453 dpm/nmole). The reaction was incubated for 30 min at 37°C.

* sonicated in buffer

Furthermore, results summarized in Table 12 obtained with diacyl-[^{14}C]-glycerol substantiate the conclusion that a direct incorporation of diglyceride into ethanolamine phosphatide occurs. Mild alkaline hydrolysis of the labelled phosphatidylethanolamine fraction yielded glycerophosphorylethanolamine as the sole labelled product (R_f = 0.63 with chromatography System 6). Again labelling phosphatidylethanolamine from diacyl-[^{14}C]-glycerol required no added cofactors and proceeded in the presence of ethylenediaminetetraacetic acid. There was no increase in phosphatidylethanolamine formation when serine was added along with Mg^{++} and CTP. When nucleotide and Mg^{++} were added, a new component appeared which ran close to the origin (the slow-moving component) when lipid extracts were chromatographed in chloroform: methanol:water (65:25:4 by vol., System 1). After elution of this component by Bligh and Dyer extraction (150) of the silica gel G, it was subjected to mild alkaline hydrolysis. Identification of the hydrolysis products, glycerophosphorylserine and α-glycerophosphate with chromatography system 6 indicated that, with Mg^{++} and CTP as additives in the incubation mixture, both phosphatidylserine and phosphatidic acid had formed and together accounted for the slow-moving component (cf. Table 13). When Mg^{++} and ATP were added in the absence of CTP, only labelled phosphatidic acid appeared in the slow-moving component (cf. Table 13). Nucleotide and Mg^{++} addition caused the appearance of [^{14}C]-phosphatidylglycerol probably via labelled phosphatidic acid formation which accumulates under these conditions (cf. Table 12 and 13). The accumulation of phosphatidic acid did not, however, favour labelling of phosphatidylethanolamine.

Table 12

The Incorporation of diacyl-[^{14}C]-
Glycerol into Labelled Phosphoglycerides

Conditions	Counts Recovered in Products			
	slow-moving component	PG	PE	CL
+ 5 mM EDTA	0	0	2,440	0
+ 2.8 mM ATP	1,617	0	2,508	252
+ 2.8 mM ATP + 10 mM Mg^{++}	11,145	3,039	2,156	574
+ 0.4 mM CTP + 10 mM Mg^{++}	7,957	1,821	1,826	617
+ 0.4 mM CTP + 10 mM Mg^{++} + 20 mM Serine	5,359	1,223	2,457	482

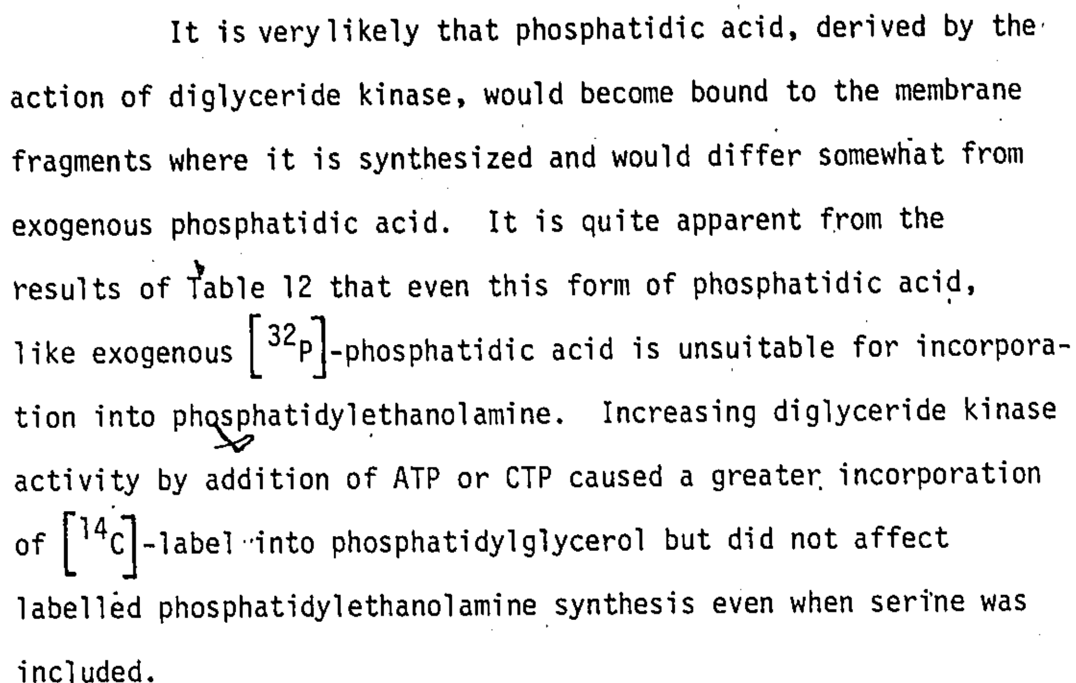
The reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 1.78 mg *E. coli* particulate protein, 0.1% Triton X-100, 30,000 dpm diacyl-[^{14}C]-glycerol (sp. act. = 1,220 dpm/nmole) and additives as indicated. The reaction was incubated for 30 min at 37°C.

Table 13

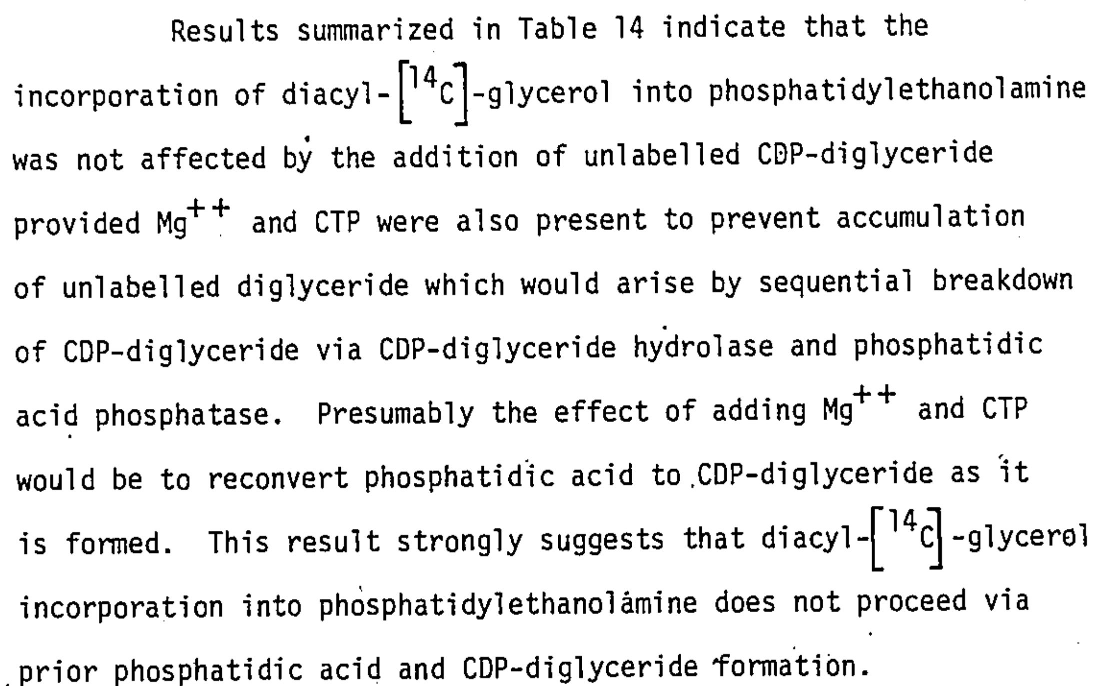
Analysis of the Slow-Moving Component
Labelled from diacyl-[^{14}C]-Glycerol

Conditions	R_f Values *	
	Peak 1	Peak 2
2.8 mM ATP + 10 mM Mg^{++}	-	0.28
0.4 mM CTP + 10 mM Mg^{++}	0.14	0.24
2.8 mM ATP + 0.4 mM CTP + 10 mM Mg^{++}	0.13	0.26
Glycerophosphorylserine standard	0.13	
α-Glycerophosphate standard		0.26

* The products were obtained from mild alkaline hydrolysis of the slow-moving component labelled from diacyl-[^{14}C]-glycerol (cf. Table 12) and analysed by paper chromatography with phenol:water (5:2 v/v) as solvent.



It is very likely that phosphatidic acid, derived by the action of diglyceride kinase, would become bound to the membrane fragments where it is synthesized and would differ somewhat from exogenous phosphatidic acid. It is quite apparent from the results of Table 12 that even this form of phosphatidic acid, like exogenous $[^{32}P]$-phosphatidic acid is unsuitable for incorporation into phosphatidylethanolamine. Increasing diglyceride kinase activity by addition of ATP or CTP caused a greater incorporation of $[^{14}C]$-label into phosphatidylglycerol but did not affect labelled phosphatidylethanolamine synthesis even when serine was included.



Results summarized in Table 14 indicate that the incorporation of diacyl-$[^{14}C]$-glycerol into phosphatidylethanolamine was not affected by the addition of unlabelled CDP-diglyceride provided Mg^{++} and CTP were also present to prevent accumulation of unlabelled diglyceride which would arise by sequential breakdown of CDP-diglyceride via CDP-diglyceride hydrolase and phosphatidic acid phosphatase. Presumably the effect of adding Mg^{++} and CTP would be to reconvert phosphatidic acid to CDP-diglyceride as it is formed. This result strongly suggests that diacyl-$[^{14}C]$-glycerol incorporation into phosphatidylethanolamine does not proceed via prior phosphatidic acid and CDP-diglyceride formation.

Table 14

The Effect of CDP-diglyceride on the
Incorporation of diacyl-[14C]-Glycerol
into Labelled Phosphoglycerides

Conditions	Counts Recovered in Phosphatidylethanolamine
standard conditions	4682
+ 0.4 mM CDP-diglyceride	3764
+ 0.4 mM CDP-dg + 10 mM Mg^{++} + 0.4 mM CTP	5052

The standard reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 5.0 mg *E. coli* homogenate protein 0.1% Triton X-100, and 24,845 dpm of diacyl-[14C]-glycerol (sp. act. = 1,220 dpm/nmole). The reaction was incubated for 30 min at 37oC.

D. The Effect of Exogenous Phosphoglycerides on the Incorporation of [^{14}C]-Serine into Phosphoglycerides

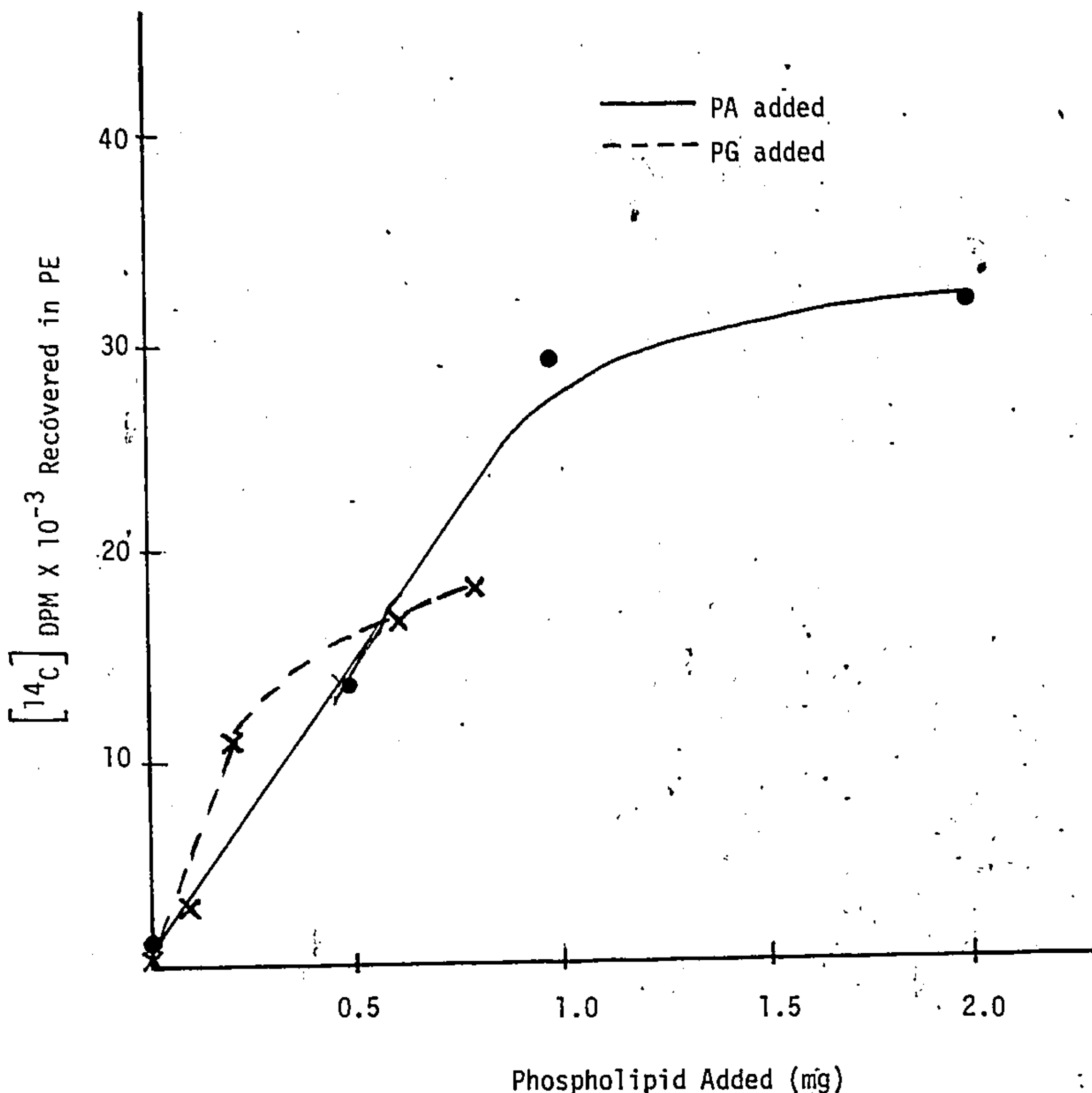
Results in the present thesis have indicated that diglyceride incorporates quite readily into phosphatidylethanolamine and that one source of this neutral lipid is phosphatidic acid. Accordingly there is an enzyme in *E. coli* capable of removing the phosphate group from phosphatidic acid, albeit, its more predominant activity is against the lyso analogue under the conditions assayed (76); furthermore, labelled diglyceride accumulates as one product when [^{14}C]-phosphatidic acid is incubated with *E. coli* preparations under conditions which allow phosphatidylethanolamine labelling as well.

Raetz has suggested that polyglycerophosphatides, known to donate their *sn*-glycero-1-phosphate group to a class of periplasmic oligosaccharides of *E. coli* discussed previously (83), can lead to diglyceride formation as a by-product of this reaction. The oligosaccharide formation is stimulated by UDP-glucose and one would expect that this nucleotide sugar would also stimulate diglyceride formation from polyglycerophosphatides if the pathway suggested by Raetz is correct. Since diglyceride readily incorporates into phosphatidylethanolamine, this lipid should acquire the label from [^{14}C]-phosphatidylglycerol and/or [^{14}C]-cardiolipin under the right conditions.

Results illustrated in Figure 8 indicate that exogenous phosphatidic acid and phosphatidylglycerol significantly stimulate the incorporation of [^{14}C]-serine into phosphatidylethanolamine. The result with phosphatidic acid was expected on the basis of the hydrolysis of this lipid to diglyceride. Exogenous phosphatidylethanolamine and phosphatidylcholine were completely ineffective whereas cardiolipin

Figure 8

The Effect of Exogenous Phosphatidic Acid and Phosphatidylglycerol on the Incorporation of [^{14}C]-Serine in Labelled Phosphatidylethanolamine



The reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 10 mM $MgCl_2$, 0.4 mM CTP, 0.1% Triton X-100, 4.66 mg *E. coli* homogenate protein, 199,121 dpm [^{14}C]-serine (sp. act. = 151 mCi/mmole), and phosphatidic acid or phosphatidylglycerol in the amounts indicated. The reaction was incubated for 30 min at 37°C.

just as phosphatidylglycerol stimulated this incorporation to a significant degree (Table 15).

The results just described indicate that polyglycerophosphatides could stimulate the incorporation of $[^{14}C]$-serine into phosphatidylethanolamine. This stimulation could be explained by some activation effect of the polyglycerophosphatides on the enzyme(s) responsible for phosphatidylethanolamine synthesis or by a precursor role of these lipids for the ethanolamine phosphatide. To test the latter possibility, $[^{32}P]$- and $[^{14}C]$-acyl polyglycerophosphatides were incubated with *E. coli* homogenate fraction under different conditions. Preliminary results obtained in this laboratory have indicated a conversion of $[^{14}C]$-phosphatidylglycerol but not $[^{32}P]$-phosphatidylglycerol to labelled phosphatidylethanolamine. $[^{14}C]$-cardiolipin was also incorporated to a lesser extent but $[^{32}P]$-cardiolipin was not. UDP-glucose did not stimulate this conversion.

On the basis of these results one can suggest that diglyceride moieties for phosphatidylethanolamine formation may arise not only via phosphatidic acid but from polyglycerophosphatides also. However, no definite conclusions can be made at this point regarding the possible pathways involved in the conversion of polyglycerophosphatides to phosphatidylethanolamine.

E. The Formation of $[^{14}C]$-Phosphatidylethanolamine from an Endogenous Labelled Cytosol Factor

Since the incorporation of labelled diglyceride into phosphatidylethanolamine did not seem to involve its prior conversion to phosphatidic

Table 15

The Effect of Exogenous Phospholipids on the Incorporation of [^{14}C]-Serine into Phosphoglycerides

Conditions	Counts Recovered in Products	
	PS	PE
standard conditions	0	1605
+ 1 mg cardiolipin	1592	4777
+ 1 mg phosphatidylglycerol	1850	5889
+ 1 mg phosphatidylethanolamine	0	0
+ 1 mg phosphatidylcholine	0	0

The standard reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 1.52 mg *E. coli* particulate protein, 10 mM $MgCl_2$, 0.4 mM CTP, 0.1% Triton X-100, 216,200 dpm [^{14}C]-serine (sp. act. = 153 mCi/mmole) and additives as listed. The reaction was incubated for 30 min at 37°C.

The exogenous phospholipids were added to each appropriate tube as a chloroform solution. Triton X-100 was then added and mixed with the chloroform. The chloroformed was evaporated off by a gentle flow of nitrogen and to this micellized lipid residue, buffer, cofactors, and enzyme were added.

acid or to CDP-diglyceride and since none of the cofactor additives tried, such as CTP, Mg^{++}, serine, ethanolamine, phosphoserine, phosphoethanolamine, and CDP-ethanolamine stimulated this incorporation, some endogenous cofactor(s) donating a phosphoserine or phosphoethanolamine group must have been present in the *E. coli* extracts used which promoted the formation of the ethanolamine and serine phosphatides.

Although incorporation of $[^{14}C]$-diglyceride proceeded with particulate fractions, the endogenous cofactor might well be formed in the cytosol fraction and be most abundant as a soluble component of the cell. Evidence supporting this was sought.

E. coli cells were grown to the exponential phase in a medium containing $[^{14}C]$-serine. The cells were disrupted by ultrasonication and a labelled cytosol fraction was prepared by centrifugation for 2 hours at 200,000 X g. The cytosol fraction, after extensive dialysis, was then incubated with particulate fraction prepared from cells grown in a medium without isotope. Results summarized in Table 16 indicate that the label from cytosol can be transferred to phosphatidylserine and to phosphatidylethanolamine by particulate fractions but not to phosphatidylglycerol and that this conversion is stimulated by added diglyceride. The evidence did strongly support the presence of a labelled non-dialysable cytosolic cofactor possibly supplying phosphoserine or phosphoethanolamine groups to the ethanolamine phosphatide and serine phosphatide fraction.

Table 16

The Formation of [^{14}C]-Phosphatidylethanolamine from [^{14}C]-Serine-Labelled Cytosol and *E. coli* Particulate Fraction

Conditions	Product Formed (DPM)	
	PS	PE
standard conditions	191	1451
+ 0.1 mM dipalmitin	1416	6711

The standard reaction mixture contained in 2 mL 0.1 M potassium phosphate buffer pH 7.4, 1.27 mg *E. coli* particulate protein 0.1% Triton X-100, 119,050 dpm [^{14}C]-serine-labelled 200,000 X g supernatant. The reaction was incubated for 30 min at 37°C.

F. Studies on the Presence and Characterization of a Cytosolic Cofactor for the Synthesis of Phosphatidylserine and Phosphatidylethanolamine

Cytosol fraction was prepared from $[^{14}C]$-serine labelled cells as previously described. After dialysis it was analysed with a Sephadex G-50 (fine) column. The procedures involved have been described in the Material and Methods section. A graph of the elution pattern is given in Figure 9. Three major protein peaks were eluted but only one designated A contained radioisotope in significant amounts. This peak was contained in the void volume. On one occasion the "void volume" peak was followed by a second peak just barely retained by the column but this result could not be reproduced.

When peak A material was lyophilized and extracted by Bligh and Dyer extraction (150), it was found to contain the usual *E. coli* lipids accounting for over 6-7% of the total label. The remainder of the label was probably associated with the protein but this point was not checked. When tested with particulate fraction for cofactor activity, material from peak A donated only a very small number of counts to the phosphatidylethanolamine fraction (Table 17). However, this result was reproducible with three different labelled A fractions analysed. Addition of diglyceride stimulated the formation of labelled phosphatidylethanolamine. Following Bligh and Dyer extraction (150) to remove lipids, fraction A (recovered in the aqueous phase), was subjected to mild alkaline hydrolysis and the hydrolysis products

Figure 9

Elution Profile of the [^{14}C]-Serine-Labelled Cytosolic Protein Fraction on Sephadex G-50

The material added to the column was a 200,000 X g supernatant from [^{14}C]-serine-endogenously labelled *E. coli* cells. The Absorbance was read at 280 nm ●——● ; the ^{14}C DPM are represented by ●---● .

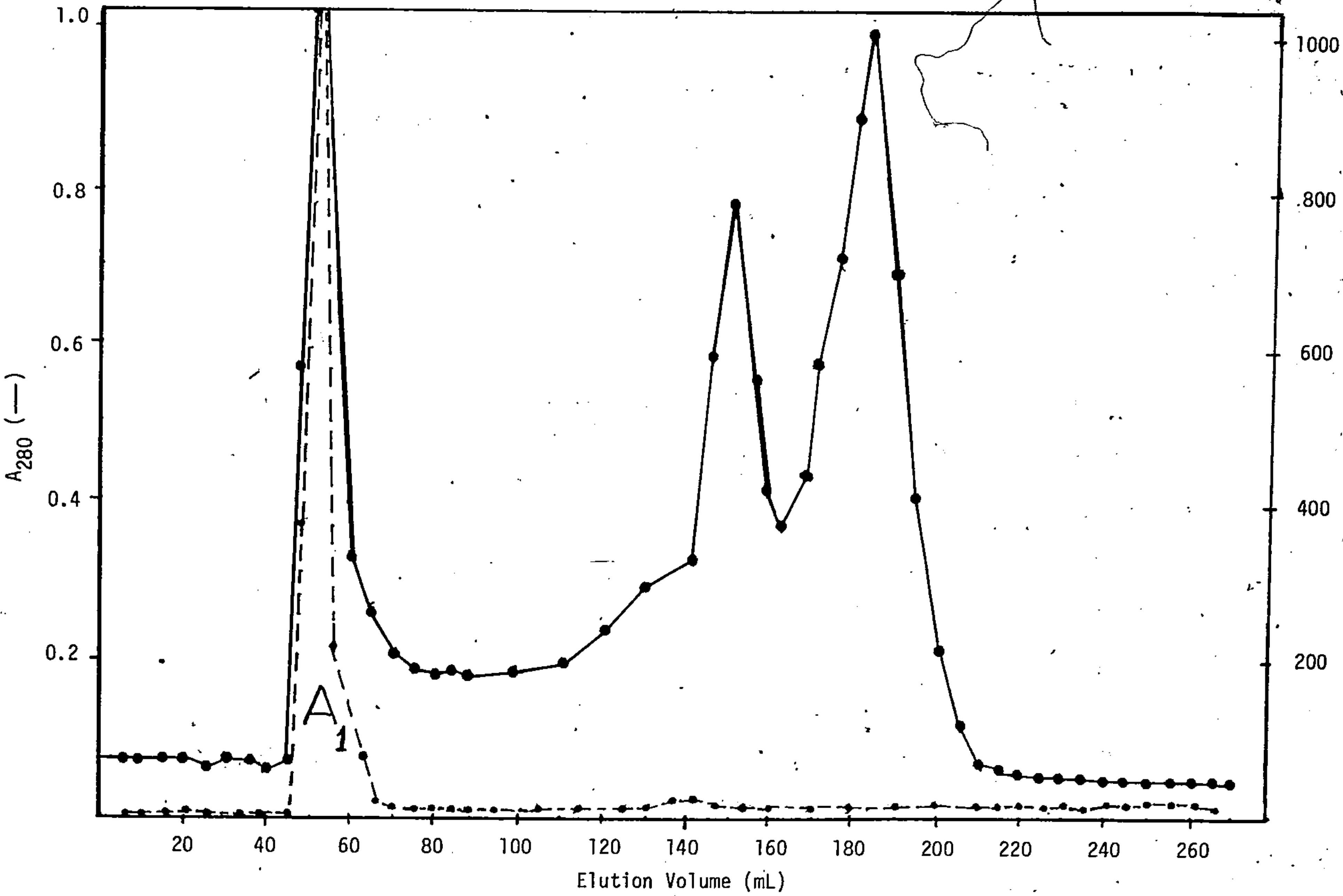


Figure 9

Table 17

The Formation of [^{14}C]-Phosphatidylethanolamine
from [^{14}C]-Serine-Labelled Protein Peak A
and *E. coli* Particulate Fraction

Conditions	Product Formed (DPM) [^{14}C]-PE
standard conditions	241
+ 1 mM 1,2 dipalmitin	475
+ 2 mM 1,2 dipalmitin	417

The standard reaction mixture contained in 1.6 mL 0.1 M potassium phosphate buffer pH 7.4, 1.6 mg *E. coli* particulate protein, 0.1% Triton X-100, and 50,000 dpm [^{14}C]-serine-labelled protein peak A*. 1,2-Dipalmitin dissolved in chloroform was added to the tubes and evaporated off by a stream of N_2 before other components were added. The reaction was incubated for 30 min at 37°C.

* Peak A contains lipoprotein material and in the amount of protein fraction used 3259 DPM of phosphatidylethanolamine were recovered. The DPM reported in the table represent a net increase in phosphatidylethanolamine labelling in the presence of active enzyme.

were analysed by paper chromatography with system 6 (cf. Table 18). Two major products were obtained, phosphoserine (R_f = 0.10) and unidentified material which ran at the solvent front. Two minor peaks were also seen corresponding to glycerophosphate (R_f = 0.23) and phosphoethanolamine (R_f = 0.33).

To further identify the substance which eluted in the void volume on Sephadex G-50 columns, another cytosol fraction was prepared from cells that had been grown for 7 hours in 300 mL media containing 100 uCi $[^{14}C]$-serine and 5 mCi $[^{32}P]$-orthophosphate.

The cytosol fraction was then separated on a Sephadex G-50 column and Peak A material was isolated as usual (cf Figure 10). It contained both $[^{14}C]$ and $[^{32}P]$ label. After lyophilization and dissolution in 10 mL water, this material was passed through another column prepared with Sephadex G-150 and eluted at a rate of 0.5 mL per min. The elution pattern is illustrated in Figure 11. Two major protein peaks were obtained which were $[^{32}P]$-labelled and $[^{14}C]$-labelled. However, the isotope elution pattern was monitored only for the $[^{32}P]$ label. Peak A_1 contained the material eluted with 55-90 mL buffer. Peak A_2 contained the material eluted with 160-200 mL of buffer. Results summarized in Table 19 indicate that both $[^{14}C]$ and $[^{32}P]$ label from peak A_1 could be transferred to phosphatidylethanolamine in the presence of diglyceride and that the process was inhibited slightly by Mg^{++}.

Table 18

Analysis of the Hydrolysis Products of the [14C]-Serine-Labelled Protein Peak A from Sephadex G-50

Component *	R_f Values
1	0.10
2	0.23
3	0.33
phosphoserine standard	0.11
phosphoethanolamine standard	0.34
glycerophosphorylethanolamine standard	0.63
glycerophosphorylserine standard	0.08

* These components were obtained by mild alkaline hydrolysis of the [14C]-serine-labelled aqueous phase following Bligh and Dyer extraction (150) of lipids from the [14C]-serine Peak A from Sephadex G-50. The solvent used was phenol/water (5:2 w/w).

Figure 10

Elution Profile of the [^{14}C]-Serine- and [^{32}P]-Labelled Cytosolic Fraction on Sephadex G-50

The material added to the column was a 200,000 X g supernatant from [^{14}C]-Serine- and [^{32}P]-endogenously-labelled *E. coli* cells. The Absorbance was read at 280 nm •——•; only the [^{32}P] DPM are drawn for the sake of clarity •---•.

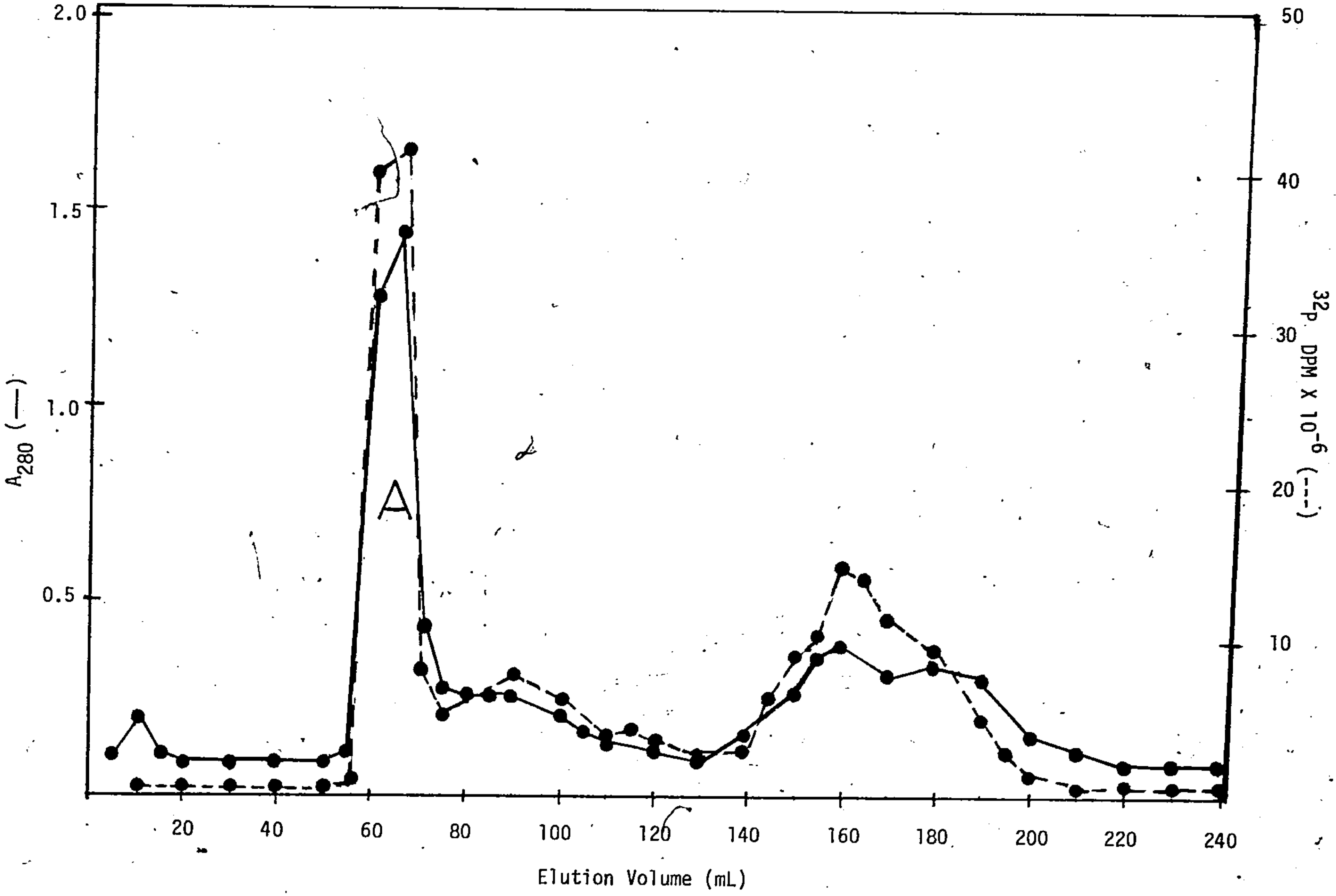


Figure 10

Figure 11

Elution Profile of the [^{14}C]-Serine- and [^{32}P]-Labelled Material from Peak A on Sephadex G-150

The material added to the column was Peak A obtained from Sephadex G-50 (Figure 10). The Absorbance was read at 280 nm ●——●; the [^{32}P] DPM only are drawn ●- -●.

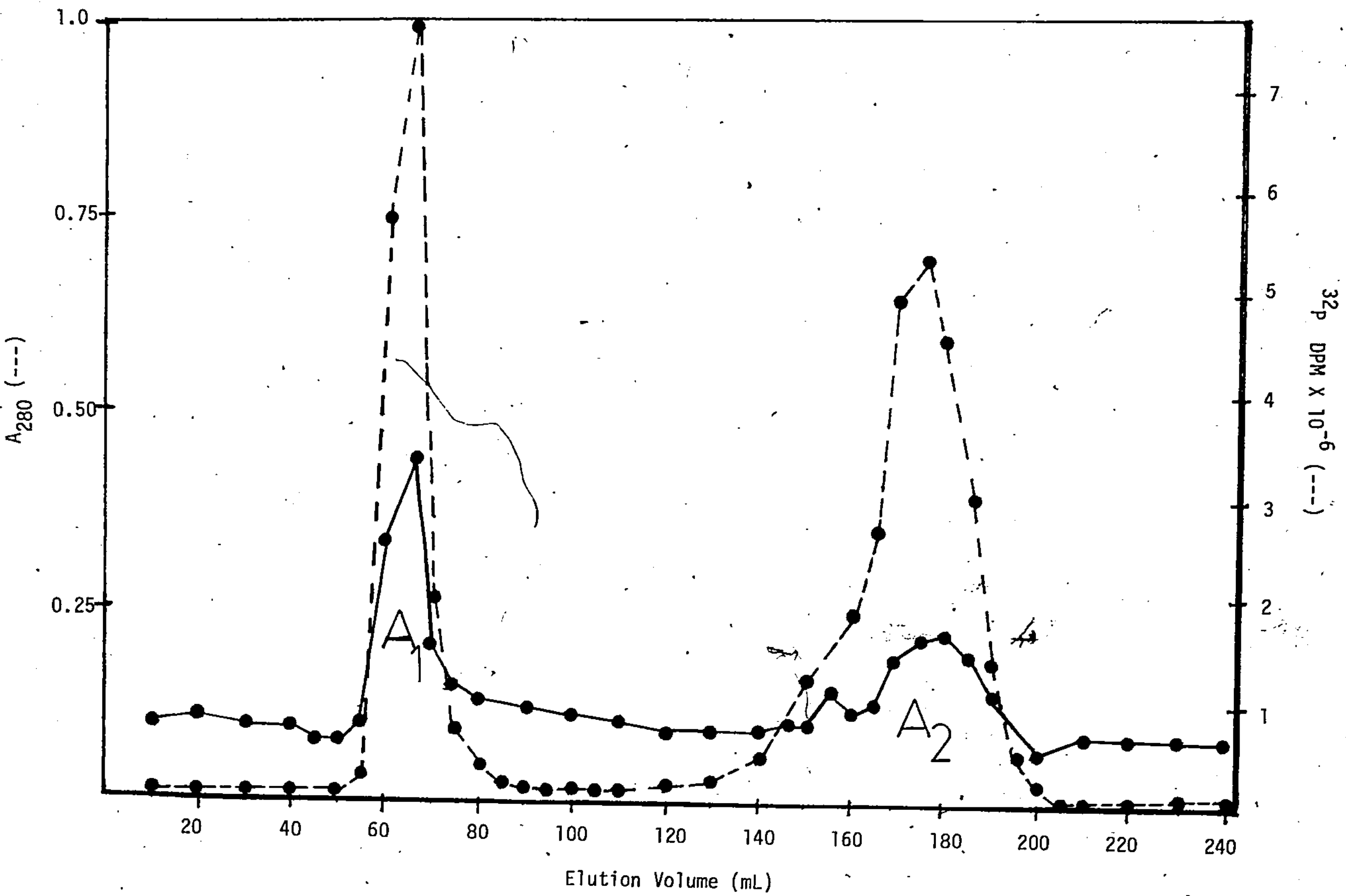


Figure 11

Table 19

The Formation of Labelled Phosphatidylethanolamine from $[^{14}C]$-Serine- and $[^{32}P]$-Labelled Peak A_1 from Sephadex G-150

Conditions	Product Formed DPM	
	$[^{14}C]$-PE	$[^{32}P]$-PE
standard conditions	2375	828
+ 10 mM Mg^{++}	1784	445

The standard reaction mixture contained in 1.6 mL 0.1 M potassium phosphate buffer pH 7.4, 1.6 mg *E. coli* particulate protein, 100,000 dpm $[^{14}C]$-serine and $[^{32}P]$-labelled protein peak A_1 from Sephadex G-150, 2 mM dipalmitin, and 0.1% Triton X-100. The reaction was incubated for 30 min at 37°C.

It may be recalled, however, (cf Table 9) that diglyceride incorporation does not require Mg^{++} and proceeds in the presence of EDTA.

Peak A_2 material did transfer a small amount of label to phosphatidylethanolamine when tested just after isolation but appeared to be labile and reproducible results could not be obtained.

Results summarized in Table 20 indicate that material from peak A_1 was susceptible to trypsin treatment and lost about half of its cofactor activity after 30 min treatment with the proteolytic enzyme.

Table 20

The Effect of Trypsin on the Incorporation of [^{14}C]-Serine- and [^{32}P]-Labelled Peak A_1 into Labelled Phosphatidylethanolamine

Conditions	Product Formed DPM [^{14}C]-PE	[^{32}P]-PE
standard conditions	1983	1845
with trypsin-treated* peak A_1	1241	1004

The standard reaction mixture contained in 1.6 mL 0.1 M potassium phosphate buffer pH 7.4, 1.6 mg *E. coli* particulate protein, 0.1% Triton X-100, and 500,000 dpm of the [^{14}C]-Serine and [^{32}P]-labelled Peak A_1. The reaction was incubated for 30 min at 37°C.

*description of trypsin treatment on page 49.

DISCUSSION

The published pathway (58,61,62) for synthesis of phosphatidylethanolamine involves the conversion of phosphatidic acid to CDP-diglyceride which is in turn the common precursor of phosphatidylserine and phosphatidylglycerophate. The phosphatidylserine is decarboxylated finally to form phosphatidylethanolamine. The evidence for this pathway is not all unambiguous. Pulse labelling and chase studies of cells don't always indicate without doubt the pathway that is used for the formation of a product under study. All that these studies have shown in fact is that phosphatidic acid, CDP-diglyceride, and phosphatidylserine precede formation of other phosphatides in *E. coli*. Furthermore, the identification of these transient intermediates was tentative in some cases at least (82).

Genetic information has supported the fact that mutants defective in their phosphatidylserine synthetase have a decreased phosphatidylethanolamine content. On the other hand, some mutants unable to form phosphatidylethanolamine have a normal phosphatidylserine synthetase and decarboxylase activities (92). Perhaps the most definite proof that the published pathway for synthesis of phosphatidylethanolamine really exists is the fact that phosphatidylserine synthetase, an enzyme which forms phosphatidylserine from CDP-diglyceride and serine has been isolated and well characterized (87,88). This finding in itself however does not prove that the pathway is operational or that it is the

only pathway. It only indicates that *E. coli* has the enzymatic equipment for the synthesis of phosphatidylethanolamine to proceed in this particular manner. Not all enzymes found in nature, however, have a prescribed role. Phospholipase A of *E. coli* is a case in point of a well characterized enzyme with no known function.

Results in this thesis point to the existence of an alternative pathway for phosphatidylethanolamine synthesis in *E. coli*. First, exogenous $[^{32}P]$-labelled phosphatidic acid was found to donate its label not for phosphatidylethanolamine synthesis but for polyglycerophosphatide formation exclusively. In the process, CDP-diglyceride must have been formed since unlabelled CDP-diglyceride addition blocked this conversion of $[^{32}P]$-phosphatidic acid to labelled phosphatidylglycerol. This result could be explained in several ways. CDP-diglyceride formation is believed to be a rate limiting step (83) and therefore only very minute amounts would be available for the phosphatidylserine and phosphatidylglycerolphosphate synthetases. If the latter enzyme had a much larger affinity for the liponucleotide substrate than the former enzyme, little or no phosphatidylserine and phosphatidylethanolamine synthesis would occur unless CDP-diglyceride were added exogenously. It was found with dialysed homogenates, however, that with CDP-diglyceride added to the medium, labelled phosphatidylserine synthesis from $[^{14}C]$-serine proceeded much more readily than did labelled phosphatidylglycerol formation from $[^{14}C]$-α-glycerophosphate (Figure 7). This means that the affinity of the phosphatidylserine synthetase for the liponucleotide may actually be better than that for the

phosphatidylglycerophosphate synthetase. It is unlikely that the poorer incorporation of [^{14}C]-α-glycerophosphate is due to a greater dilution by endogenous precursor since dialysed preparations were used and the endogenous glycerophosphate content is negligible in any case (76).

Unfortunately, no detailed kinetic parameters for the purified synthetases have been published (61,87,88,99) which would allow a more complete comparison of the enzymes with respect to their liponucleotide requirement.

Another explanation for the lack of [^{32}P]-phosphatidic acid incorporation into phosphatidylethanolamine would be that exogenous phosphatidic acid leads to a pool of CDP-diglyceride which is accessible only to the polyglycerophosphatide synthesis enzymes. Phosphatidylethanolamine formation would proceed only from endogenous phosphatidic acid or CDP-diglyceride. This explanation could be partly true but not entirely satisfactory since exogenous CDP-diglyceride greatly stimulated [^{14}C]-serine incorporation into phosphatidylethanolamine. Very likely then, exogenous CDP-diglyceride is a suitable form of liponucleotide for the phosphatidylserine synthetase. Also [^{14}C]-phosphatidic acid readily incorporated into phosphatidylethanolamine in a process which also involves labelled diglyceride production as a concurrent by-product. It seems that [^{32}P]-phosphatidic acid did not incorporate into phosphatidylethanolamine because it is only the diglyceride moiety of exogenous phosphatidic acid that is used for phosphatidylethanolamine synthesis. Accordingly,

$[^{14}C]$-diglyceride was found to readily incorporate into phosphatidylethanolamine. With homogenate dialysed 18-20 hours, factors such as CTP, Mg^{++}, serine, phosphoserine, phosphoethanolamine, and CDP-ethanolamine did not stimulate the incorporation which proceeded even in the presence of EDTA. The addition of ATP or CTP together with Mg^{++} stimulated the formation of phosphatidylglycerol without affecting the formationof phosphatidylethanolamine. The $[^{14}C]$ entry probably occurred via diglyceride kinase activity, the product of this reaction being then converted to CDP-diglyceride.

It seems that even this form of phosphatidic acid and CDP-diglyceride derived from exogenous diglyceride is unsuitable for phosphatidylethanolamine synthesis. The incorporation of $[^{14}C]$-diglyceride into phosphatidylethanolamine was only slightly diminished by unlabelled CDP-diglyceride addition. If CTP and Mg^{++} were added together with CDP-diglyceride, the liponucleotide had no diminishing effect. The results indicate that diglyceride incorporation into phosphatidylethanolamine does not proceed via phosphatidic acid and CDP-diglyceride. The small inhibition by the liponucleotide is probably due to its hydrolysis to phosphatidic acid and CMP. Phosphatidic acid is in turn hydrolyzed to diglyceride and isotope dilution ensues. CTP and Mg^{++} addition must reconvert phosphatidic acid product into CDP-diglyceride as it is formed.

The results discussed thus far indicate that if CDP-diglyceride is used for phosphatidylethanolamine synthesis it must be exogenous,

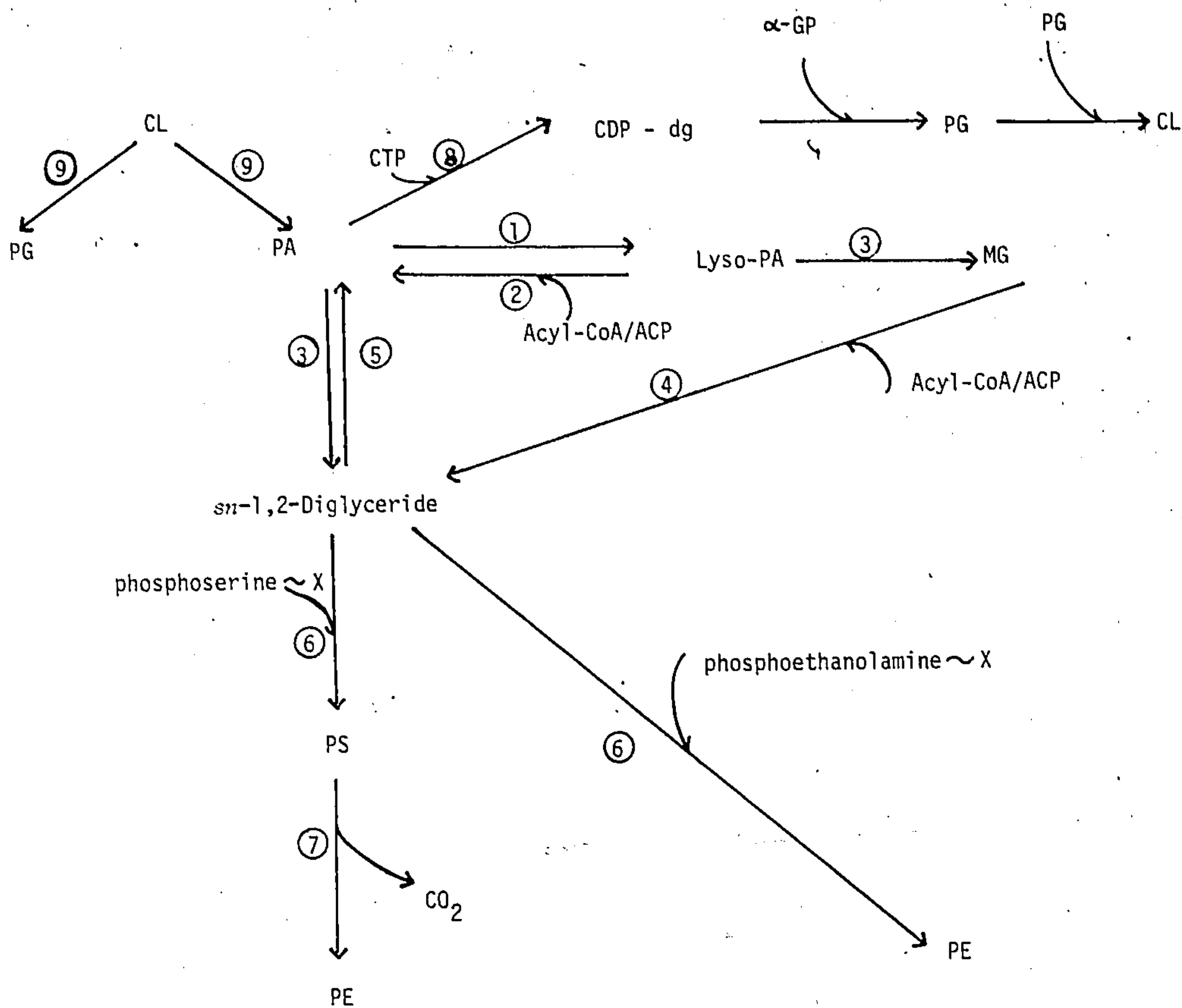
or derived from an endogenous pool of phosphatidic acid quite different than the one used for phosphatidylglycerol and cardiolipin synthesis.

Since the incorporation of diglyceride did not seem to depend on cofactor additives, an endogenous factor supplying the phosphorylserine and/or phosphorylethanolamine moieties for phosphatidylethanolamine formation must have been present in our extracts. Accordingly, cytosol fractions labelled with $[^{14}C]$-serine were found to incorporate their label into phosphatidylethanolamine, this incorporation being stimulated by added diglyceride. The endogenous cofactor detected was non-dialysable but some dialysable labelled cofactor may have been lost. This point was not investigated. Further characterization of the non-dialysable cofactor was attempted by labelling *E. coli* cells with $[^{14}C]$-serine, by preparing a cytosol fraction from such cells and after dialysis, by subjecting this fraction to Sephadex G-50 chromatography.

Material eluted with the void volume contained protein and lipid and was $[^{14}C]$-labelled. Incubation of this "lipoprotein" fraction with fresh particulate preparation resulted in a very limited but reproducible transfer of label to phosphatidylethanolamine which was stimulated by diglyceride addition. The material did yield $[^{14}C]$-phosphoserine upon mild alkaline hydrolysis and on this basis one can suggest that a protein bound phosphoserine donor is present in the soluble fraction of the cell. It may be associated with membranes as well but this point has not yet been investigated. The material eluting with the void volume on Sephadex G-50 could be doubly-labelled

if prepared from cytosol fractions isolated from cells grown in media containing [^{32}P]-orthophosphate and [^{14}C]-serine. When this doubly-labelled material was further analysed on Sephadex G-150 two peaks of radioactive components were isolated, one corresponding to material recovered with the void volume (component A_1), and the other being retained somewhat (component A_2). Both peaks were proteinaceous, and the void volume peak contained lipids which were labelled. Both peaks gave some label to phosphatidylethanolamine but only material from peak A_1 seemed sufficiently stable to give consistent results. The other material from peak A_2 appeared to be labile and lost its donor ability within a week after isolation even if stored at -20°C. Peak A_1 material gave both labels to phosphatidylethanolamine and this transfer was stimulated by diglyceride. Again results pointed to the presence of a bound form of phosphoserine and/or phosphoethanolamine which served to form phosphatidylethanolamine in the presence of diglyceride. The precise nature of this complex is unknown, however, trypsin did inactivate the complex to some extent which may indicate a phosphorylserine- (or phosphorylethanolamine-) protein complex of some sort, the protein moiety being of some importance for activity.

On the basis of the results obtained, Scheme 4 can be proposed for the synthesis of phosphoglycerides. Results presented in Fig. 5 indicate that reaction 1 does occur in *E. coli*. Both the lyso product and the fatty acid product have been characterized. Reaction 2 is also known to occur (162) and combined with reaction 1 would offer a means of changing phosphoglyceride fatty acid composition at the phosphatidic



Scheme 4

Proposed Pathway for the Synthesis of Phosphoglycerides in *E. coli*

acid level. For example such a mechanism might be important for retailoring of phosphatidic acid derived from diglyceride kinase activity (81) or from cardiolipin phosphohydrolase activity (105,138). Reaction 3 occurs quite readily in coliform extracts. In fact, it has been reported that the phosphatidic acid phosphatase of *E. coli* acts more readily on lysophosphatidic acid than on phosphatidic acid (76). Reaction 5 also occurs in *E. coli* (54) and together with reactions 1-4 may assist in the retailoring of diglyceride moieties of phosphoglycerides. Reactions 4 and 7-9 of the sequence have been well characterized (61,96,97,105,138). The evidence for reaction 6 has been presented in this thesis. The phosphoserine (and perhaps phosphoethanolamine) are proposed to be partly bound to protein in a form allowing them to serve as substrates for phosphatidylethanolamine synthesis. The binding need not be covalent. This does not mean that other forms of these substrates do not serve. Our results, however, have not been able to show this definitely. Recently, results in our laboratory have indicated that if homogenates are permitted to autolyse for 30-40 min at room temperature prior to extensive dialysis (25-30 hours at 4°C), incorporation of [^{14}C]-diglyceride can be stimulated by the addition of 15 mM - 60 mM phosphoserine and phosphoethanolamine with CTP and Mg^{++}. These cofactors have no effect if homogenates are used which were not allowed to autolyse. Autolysis may cause release of the bound cofactors and these then become dialysable or alternately may cause their hydrolysis at least partly such that a cofactor effect becomes noticeable. Present investigations

in our laboratory are aimed at the further elucidation of the phosphoserine-phosphoethanolamine donors. Autolysed and fresh homogenates are being tested for their ability to incorporate the labels from $[^{32}P]$- and $[^{14}C]$-phosphoserine, phosphoethanolamine, and their nucleotide analogues CDP-serine and CDP-ethanolamine into phosphatidylethanolamine and into the materials of the peaks designated A_1 and A_2. These investigations should shed more light on the mechanism of reaction 6 and the bound form of the phosphoryl base donor(s).

In our present study, another possible phosphoethanolamine donor was investigated. This was the lipopolysaccharide fraction of *E. coli* which is known to contain ethanolamine pyrophosphate as a constituent molecule. However, when incubated with *E. coli* extracts, this fraction did not enhance the incorporation of $[^{14}C]$-diglyceride into phosphatidylethanolamine.

The use of mutants defective in their phosphatidylethanolamine synthesizing ability but containing normal phosphatidylserine synthetase and decarboxylase activities (92) should also help clarify the possible role of reaction 6 in the synthesis of phosphatidylethanolamine in intact *E. coli* cells and in *E. coli* cell extracts.

CLAIM TO ORIGINALITY

A new mechanism for phosphatidylethanolamine synthesis in *E. coli* has been detected. This mechanism involves the direct incorporation of diglyceride into phosphatidylethanolamine involving an endogenous phosphoserine and/or phosphoethanolamine donor which is protein-associated. This newly discovered route is not involved in the formation of polyglycerophosphatides. The latter requires CDP-diglyceride as phosphatidyl donor and does not use diglyceride directly.

BIBLIOGRAPHY

1. S. DePetris (1967) J. Ultrastruct. Res. 19:45-83.

2. R.G.E. Murray, P. Steed, and H.H. Elson (1965) Can. J. Microbiol. 11:547-560.

3. M.J. Osborn, P.D. Rick, V. Lehmann, E. Rupprecht, and M. Singh (1974) Ann. N.Y. Acad. Sci. 235:52-65.

4. B.D. Davis, R. Dulbecco, H.M. Eisen, H.S. Ginsberg, W.B. Wood, and M. McCarty (1973) Microbiology, 2nd ed., p. 105-146. Harper and Row, Hagerstown, Md.

5. M.J. Osborn (1969) Ann. Rev. Biochem. 38:501-538.

6. R.M. Bell, R.D. Mavis, M.J. Osborn, and P.R. Vagelos (1971) Biochim. Biophys. Acta 249:628-635.

7. D.A. White, F.A. Albright, W.J. Lennarz, and C.A. Schnaitman (1971) Biochim. Biophys. Acta 249:636-642.

8. D.B. Datta, B. Arden, and U. Henning (1977) J. Bacteriol. 131:821-829.

9. S. Tamaki, T. Sato, and M. Matasuhashi (1971) J. Bacteriol. 105:968-975.

10. L. Lieve (1974) Ann. N.Y. Acad. Sci. 235:109-129.

11. T. Nakae and H. Nikaido (1975) J. Biol. Chem. 250:7359-7365.

12. T. Muira and S. Mizushima (1969) Biochim. Biophys. Acta 193:268-276.

13. M.J. Osborn, J.E. Gander, E. Parisi, and J. Carson (1972) J. Biol. Chem. 247:3962-3672.

14. I. Yamato, Y. Anraku, K. Hirosawa (1975) J. Biochem. (Japan) 77:705-718.

15. S. Mizushima and H. Yamada (1975) Biochim. Biophys. Acta 375:44-53.

16. C.A. Schnaitman (1970) J. Bacteriol. 104:890-901.

17. J.M. DiRienzo, K. Nakamura, and M. Inouye (1978) Ann. Rev. Biochem. 47:481-532.

18. K. Hantke and V. Braun (1973) Eur. J. Biochem. 34:284-296.

19. I. Ørskov, F. Ørskov, B. Jann, and K. Jann (1977) Bacteriol. Rev. 41:667-710.

20. V. Lehmann, O. Lüderitz, and O. Westphal (1971) Eur. J. Biochem. 21:339-347.

21. J. Smit, Y. Kamio, and H. Nikaido (1975) J. Bacteriol. 124: 942-958.

22. D.A. White, W.J. Lennarz, and C.A. Schnaitman (1972) J. Bacteriol. 109:686-690.

23. M.J. Osborn, J.E. Gander, and E. Parisi (1972) J. Biol. Chem. 247:3973-3986.

24. J.E. Cronan, Jr. and E.P. Gelmann (1975) Bacteriol. Rev. 39:232-256.

25. D.F. Silbert (1975) Ann. Rev. Biochem. 44:315-339.

26. I. Haller, B. Hoehn, and U. Henning (1975) Biochemistry 14:478-484.

27. P.F. Muhlradt and J.R. Golecki (1975) Eur. J. Biochem. 51:343-352.

28. I. Van Alpen, B. Lugtenberg, R. Van Boxtel, and K. Verhoef (1977) Biochim. Biophys. Acta 466:257-268.

29. J.E. Cronan, Jr. (1968) J. Bacteriol. 95:2054-2061.

30. M. Sinensky (1971) J. Bacteriol. 106:449-455.

31. F. Peypoux and G. Michel (1970) Biochim. Biophys. Acta 218:453-462.

32. D.F. Silbert, J.E. Cronan, Jr., T.R. Beachan and M.E. Harder (1974) Fed. Proc. Fed. Am. Soc. Exp. Biol. 33:1725-1732.

33. K. Bloch and D. Vance (1977) Ann. Rev. Biochem. 46:263-298.

34. J.J. Volpe and P.R. Vagelos (1976) Phys. Rev. 56:339-417.

35. J.E. Cronan, Jr. and P.R. Vagelos (1972) Biochim. Biophys. Acta 265:25-60.

36. P. Overath, G. Pauli, and H.U. Schairer (1969) Eur. J. Biochem. 7:559-574.

37. T.K. Ray and J.E. Cronan, Jr. (1976) Proc. Natl. Acad. Sci. U.S.A. 73:4374-4378.

38. T.K. Ray and J.E. Cronan, Jr. (1976) Biochem. Biophys. Res. Comm. 69:506-513.

39. D.F. Silbert, F. Ruch, and P.R. Vagelos (1968) J. Bacteriol. 95:1658-1665.

40. G.S. Cox, E. Thomas, H.R. Kaback, and H. Weissbach (1973) Arch. Biochem. Biophys. 158:667-676.

41. J.H. Law (1971) Acc. Chem. Res. 4:199-203.

42. J.H. Law, H. Zalkin, and T. Kaneshiro (1963) Biochim. Biophys. Acta 70:143-151.

43. K. Klein, R. Steinberg, B. Frithen, and P. Overath (1971) Eur. J. Biochem. 19:442-450.

44. J. Kanfer and E.P. Kennedy (1963) J. Biol. Chem. 238:2919-2922.

45. G.F. Ames (1968) J. Bacteriol. 95:833-843.

46. L. Rothfield and D. Romeo (1971) Bacteriol. Rev. 35:14-38.

47. J.E. Cronan, Jr. (1978) Ann. Rev. Biochem. 47:163-189.

48. L.S. Milner and H.P. Kaback (1970) Proc. Natl. Acad. Sci. U.S.A. 65:683-690.

49. H.R. Kaback (1970) Ann. Rev. Biochem. 39:561-598.

50. M. Kito, M. Ishinaga, and M. Nishihara (1978) Biochim. Biophys. Acta 529:237-249.

51. E. Motjaba, B.B. Rudkin, C.J. Cutler, and P.E. Waldron (1977) J. Biol. Chem. 252:3194-3198.

52. C.B. Hirshberg and E.P. Kennedy (1972) Proc. Natl. Acad. Sci. U.S.A. 69:648-651.

53. K.Y. Hostetler, H. van den Bosch, and L.L.M. van Deenen (1972) Biochim. Biophys. Acta 260:507-513.

54. R.A. Pieringer and R.S. Kunnes (1965) J. Biol. Chem. 240:2833-2838.

55. L.M.G. Van Golde, H. Schulman, and E.P. Kennedy (1973) Proc. Natl. Acad. Sci. U.S.A. 70:1368-1378.

56. J. Starka, J. Moravova (1970) J. Gen. Microbiol. 60:251-257.

57. Y.Y. Chang and E.P. Kennedy (1967) J. Biol. Chem. 242:516-519.

58. C.R.H. Raetz and E.P. Kennedy (1972) J. Biol. Chem. 247:2008-2014.

59. C.R.H. Raetz and E.P. Kennedy (1973) J. Biol. Chem. 248:1098-1105.

60. J. Carter (1968) J. Lipid Res. 9:748-754.

61. Y.Y. Chang and E.P. Kennedy (1967) J. Lipid Res. 8:447-455.

62. J. Kanfer and E.P. Kennedy (1964) J. Biol. Chem. 239:1720-1726.

63. E.P. Kennedy (1963) Harvey Lect. 57:143-171.

64. A. Dutt and W. Dowhan (1977) J. Bacteriol. 132:159-165.

65. M. Ishinaga and M. Kito (1974) Eur. J. Biochem. 42:483-487.

66. M. Kito and L.I. Pizer (1969) J. Biol. Chem. 244:3316-3323.

67. E.C.C. Lin (1976) Ann. Rev. Microbiol. 30:535-578.

68. N. Zwaig, W.S. Kistler, and E.C.C. Lin (1970) J. Bacteriol. 102:753-759.

69. N. Zwaig and E.C.C. Lin (1966) Science 153:755-757.

70. J.E. Cronan, Jr., T.K. Ray, and P.R. Vagelos (1970) Proc. Natl. Acad. Sci. U.S.A. 65:737-744.

71. H. Okuyama, K. Yamada, Y. Kameyama, N. Kiezawa, Y. Akamatzu, and S. Nojima (1977) Biochemistry 16:2668-2673.

72. M. Ishinaga, M. Nishihara, and M. Kito (1976) Biochim. Biophys. Acta 450:269-272.

73. M. Snider and E.P. Kennedy (1977) J. Bacteriol. 130:1072-1083.

74. H. Okuyama and S.J. Wakil (1973) J. Biol. Chem. 248:5197-5205.

75. H. Okuyama, K. Yamada, H. Izizawa, and S.J. Wakil (1976) J. Biol. Chem. 251:2487-2492.

76. H. Van den Bosch and P.R. Vagelos (1970) Biochim. Biophys. Acta 218:233-248.

77. M. Kito, M. Ishinaga, M. Nishihara, M. Kata, S. Sawada, and T. Hata (1975) Eur. J. Biochem. 54:55-63.

78. T.K. Ray, J.E. Cronan, Jr., R.D. Mavis, and P.R. Vagelos (1970) J. Biol. Chem. 245:6442-6448.

79. E.G. Schneider and E.P. Kennedy (1973) J. Biol. Chem. 248:3739-3741.

80. E.G. Schneider and E.P. Kennedy (1976) Biochim. Biophys. Acta 441:201-212.

81. C.R.H. Raetz and K.F. Newman (1978) J. Biol. Chem. 253:3882-3887.

82. J. Carter and E.P. Kennedy (1966) J. Lipid Res. 7:678-683.

83. C.R.H. Raetz (1978) Micro. Rev. 42:614-659.

84. C.R.H. Raetz, W. Dowhan, and E.P. Kennedy (1976) J. Bacteriol. 125:855-863.

85. E. Tunaitis and J.E. Cronan, Jr. (1973) Arch. Biochem. Biophys. 155:420-427.

86. C.R.H. Raetz, C.B. Hirschberg, W. Dowhan, W.T. Wickner, and E.P. Kennedy (1972) J. Biol. Chem. 247:2245-2247.

87. T.J. Larson and W. Dowhan (1976) Biochemistry 15:5212-5218.

88. C.R.H. Raetz and E.P. Kennedy (1974) J. Biol. Chem. 249:5038-5045.

89. M. Ishinaga, M. Kato, and M. Kito (1974) FEBS Lett. 49:201-202.

90. A. Ohta and I. Shibuya (1977) J. Bacteriol. 132:434-443.

91. A. Ohta, I. Shibuya, B. Maruo, M. Ishinaga, and M. Kito (1974) Biochim. Biophys. Acta 348:449-454.

92. C.R.H. Raetz (1976) J. Biol. Chem. 251:3242-3249.

93. A. Ohta, K. Ohonogi, I. Shibuya, and B. Maruo (1974) J. Gen. Appl. Microbiol. 20:21-32.

94. J.E. Cronan, Jr. (1972) Nature New Biology 240:21-22.

95. E. Hawrot and E.P. Kennedy (1975) Proc. Natl. Acad. Sci. U.S.A. 72:1112-1116.

96. W. Dowhan, W.T. Wickner, and E.P. Kennedy (1974) J. Biol. Chem. 249:3079-3084.

97. M. Satre and E.P. Kennedy (1978) J. Biol. Chem. 253:479-483.

98. W.D. Nunn, D.L. Kelly, and M.Y. Stumfall (1977) J. Bacteriol. 132:526-531.

99. T. Hirabayashi, T.J. Larson, and W. Dowhan (1976) Biochemistry 15:5205-5211.

100. T.J. Larson, T. Hirabayashi, and W. Dowhan (1976) Biochemistry 15:974-979.

101. E.M. Barnes, Jr. and S.J. Wakil (1968) J. Biol. Chem. 243:2955-2962.

102. Y.Y. Chang and E.P. Kennedy (1967) J. Lipid Res. 8:456-462.

103. C. Luzon and J.P.G. Ballesta (1976) Eur. J. Biochem. 65:207-212.

104. C.B. Hirschberg and E.P. Kennedy (1972) Proc. Natl. Acad. Sci. U.S.A. 69:648-651.

105. R. Cole, G. Benns, and P.R. Proulx (1974) Biochim. Biophys. Acta 337:325-332.

106. A. Audet, R. Cole, and P.R. Proulx (1974) Biochim. Biophys. Acta 380:414-420.

107. R. Cole and P.R. Proulx (1975) J. Bacteriol. 124:1148-1152.

108. N.Z. Stanacev, Y.Y. Chang, and E.P. Kennedy (1967) J. Biol. Chem. 242:3018-3019.

109. H. van den Bosch (1974) Ann. Rev. Biochem. 43:243-277.

110. S.A. Short and D.C. White (1972) J. Bacteriol. 109:820-826.

111. E. Bright-Gaertner and P.R. Proulx (1972) Biochim. Biophys. Acta 270:40-49.

112. K.S. Cho, G. Benns, and P.R. Proulx (1974) Biochim. Biophys. Acta 337:325-332.

113. C. Rampini (1975) C.R. Acad. Sci. 281:1431-1433.

114. C. Rampini, E. Barbu, and J. Polonovski (1970) C.R. Acad. Sci. 270:882-885.

115. E. Barbu, J. Polonovski, C. Rampini, and M. Lux (1970) C.R. Acad. Sci. 270:2596-2599.

116. A. Audet, G. Nantel, and P.R. Proulx (1974) Biochim. Biophys. Acta 348:334-343.

117. R.M. Bell, R.D. Mavis, and P.R. Vagelos (1972) Biochim. Biophys. Acta 270:504-512

118. N.G. Golden and G.L. Powell (1972) J. Biol. Chem. 247:6651-6658.

119. C.K. Fung and P.R. Proulx (1969) Can. J. Biochem. 47:371-373.

120. P.R. Proulx and C.K. Fung (1969) Can. J. Biochem. 47:1125-1128.

121. H. Okuyama and S. Nojima (1969) Biochim. Biophys. Acta 176:120-124.

122. C.J. Scandella and A. Kornberg (1971) Biochemistry 10:4447-4456.

123. P. Patriarca, S. Beckerdite, and P. Elsbach (1972) Biochim. Biophys. Acta 260:593-600.

124. O. Doi, M. Ohki, and S. Nojima (1972) Biochim. Biophys. Acta 260:244-258.

125. O. Doi and S. Nojima (1973) J. Biochem. 74:667-674.

126. F.R. Albright, D.A. White, and W.J. Lennarz (1973) J. Biol. Chem. 248:3968-3977.

127. O. Doi and S. Nojima (1975) J. Biol. Chem. 250:5208-5214.

128. G. Nantel and P.R. Proulx (1973) Biochim. Biophys. Acta 316:156-161.

129. O. Doi and S. Nojima (1974) Biochim. Biophys. Acta 369:64-69.

130. P.R. Proulx, G. Nantel, and G. Baraff (1978) Can. J. Biochem. 56:319-323.

131. M. Nishijima, S. Nakaike, Y. Tamori, and S. Nojima (1977) Eur. J. Biochem. 73:115-124.

132. M. Abe, N. Okamoto, O. Doi, and S. Nojima (1974) J. Bacteriol. 119:543-546.

133. O. Doi and S. Nojima (1976) J. Biochem. (Japan) 80:1247-1258.

134. M. Ohki, O. Doi, and S. Nojima (1972) J. Bacteriol. 110:864-869.

135. R.F.A. Zwaal, B. Roelofsen, P. Confurius, and L.L. M. van Deenen (1971) Biochim. Biophys. Acta 233:474-479.

136. P.R. Proulx and L.L. M. van Deenen (1967) Biochim. Biophys. Acta 144:171-174.

137. O. Doi and S. Nojima (1971) Biochim. Biophys. Acta 248:234-244.

138. R. Cole and P.R. Proulx (1977) Can. J. Biochem. 55:1228-1232.

139. J.E. Cronan, Jr., L.J. Weissberg, and R.G. Allen (1975) J. Biol. Chem. 250:5835-5840.

140. W.D. Nunn, I.A. Meade, and B.P. Tropp (1975) J. Bacteriol. 132:526-531.

141. W.D. Nunn and J.E. Cronan, Jr. (1976) Biochemistry 15:2546-2550.

142. D.R. Leuking and H. Goldfine (1975) J. Biol. Chem. 250:4911-4917.

143. J.P. Merlie, and L.J. Pizer (1973) J. Bacteriol. 116:355-366.

144. M. Ishinaga, M. Kato, and M. Kito (1974) J. Biochem. (Japan) 75:1395-1397.

145. P.H. Patterson and W.J. Lennarz (1971) J. Biol. Chem. 246:1062-1072.

146. E.L. Thomas, H. Weissbach, and H.R. Kaback (1972) Arch. Biochem. Biophys. 150:797-806.

147. H. Weissbach, E.L. Thomas, and H.R. Kaback (1971) Biochim. Biophys. Acta 147:249-254.

148. C.R.H. Raetz, T.J. Larson, and W. Dowhan (1977) Proc. Natl. Acad. Sci. U.S.A. 74:1412-1416.

149. O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951) J. Biol. Chem. 193:265-275.

150. E.G. Bligh and W.S. Dyer (1959) Can. J. Biochem. and Physiol. 37:911-917.

151. M.O. Marshall and M. Kates (1972) Biochim. Biophys. Acta 260:558-570.

152. J. Baddiley, J.G. Buchanan, R.E. Handschumacher, and J.F. Prescott (1956) J. Chem. Soc. 2818-2823.

153. G.R. Bartlett (1959) J. Biol. Chem. 234:466-468.

154. S.F. Yang, S. Freer, and A.A. Benson (1967) J. Biol. Chem. 242:477-484.

155. P. Plackett (1967) Biochem. 6:2746-2754.

156. H.P. Chu (1949) J. Gen. Microbiol. 3:255-273.

157. J.G. Hildebrand and J.H. Law (1964) Biochemistry 3:1304-1308.

158. D.C. White and A.N. Tucker (1969) J. Lipid Res. 10:220-233.

159. C. Galanos, O. Lüderitz, and O. Westphal (1969) Eur. J. Biochem. 9:245-249.

160. H.M. Cunningham and W.F.M. Leat (1969) Can. J. Biochem. 47:1013-1020.

161. P. McPhie (1971) Methods in Enzymol. 22:23-32.

162. R.M. Bell (1975) J. Biol. Chem. 250:7147-7152.

ABSTRACT

Dialysed *E. coli* homogenate incorporated [^{14}C]- or [^{32}P]-phosphatidic acid exclusively into phosphatidylglycerol and cardiolipin under conditions which permitted incorporation of [^{14}C]-serine into phosphatidylethanolamine. Dialysed particulate fractions incorporated [^{14}C]-diglyceride exclusively into phosphatidylethanolamine in the absence of added cofactors. This incorporation was not affected by various combinations of additives such as CTP, Mg^{++}, phosphoserine, and phosphoethanolamine as well as by CDP-diglyceride, ATP, and EDTA. It appeared that phosphatidylethanolamine formation from diglyceride depended on an uncharacterized, endogenous, non-dialysable precursor. Cytosol fractions prepared from cells grown in [U-^{14}C]-serine medium were found to contain a labelled non-dialysable factor which in the presence of particulate fractions incorporated its label into phosphatidylethanolamine. This incorporation was stimulated by diglyceride addition. The factor separated with lipoprotein material on Sephadex G-50 and G-150 columns and upon mild alkaline hydrolysis yielded a labelled component which cochromatographed with phosphoserine. The results are discussed in terms of an alternative route for the synthesis of phosphatidylethanolamine in *E. coli*.